

BIOSYNTHESIS OF CARTILAGE
PROTEOGLYCANS IN CHONDROCYTE
CELL CULTURES

Sven Björnsson



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By

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In memory of my father

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LIST OF PAPERS

This thesis is based on the following papers which are referred to by their Roman numerals.

- I. Sven Björnsson and Dick Heinegård.
Isolation and culture techniques of foetal calf chondrocytes.
Biochem. J. In press.
- II. Sven Björnsson and Dick Heinegård.
Fractionation and characterization of proteoglycans isolated from chondrocyte cell cultures.
Biochem. J. In press.
- III. Ahnders Franzén, Sven Björnsson and Dick Heinegård.
Zonal rate centrifugation of proteoglycans in sucrose gradient.
Analytical Biochemistry. Submitted.
- IV. Ahnders Franzén, Sven Björnsson and Dick Heinegård.
Cartilage proteoglycan in aggregate formation. Role of link protein.
Biochem. J. In press.
- V. Sven Björnsson and Dick Heinegård.
Assembly of proteoglycan aggregates in cultures of chondrocytes from bovine tracheal cartilage.
Biochem. J. In press.
- VI. Sven Björnsson and Dick Heinegård.
Binding of link protein to proteoglycan and hyaluronic acid in cultures of chondrocytes from bovine tracheal cartilage.
To be submitted.

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ABBREVIATIONS

dwt - dry weight
gal - galactose
NANA - N-acetylneuraminic acid
SDS - sodium dodecyl sulphate
xyl - xylose

INTRODUCTION

The cartilage tissue, as well as bone, appeared early in the evolution, as the principally supporting tissue of the vertebrate body. By sharks and rays, cartilage is the only supporting tissue. Cartilage is also a predecessor to bone during the embryonic development of the skeleton of mammals. In adult life, it provides the surface layer in joints, allowing smooth articulation, in addition to its function as supporting tissue.

The important mechanical qualities of cartilage are elasticity and resistance to compression. These characteristics reflect the molecular organization of the tissue. Hyaline cartilage consists of a gel of proteoglycans entrapped in a network of collagen fibers, each component contributes about half of the dry weight of the tissue. Chondrocytes are the only cells present and contribute only a few percent of the dry weight. Both the collagen and the proteoglycans are synthesized by these cells (Vertel & Dorfman, 1979).

Each collagen molecule contains three $\alpha 1(\text{II})$ polypeptide chains. This form of collagen is designated type II, and has to date been identified only in the hyaline cartilage (Miller & Matukas, 1974) and in the vitreous body of the eye (Trelstad & Kang, 1974).

Proteoglycans are macromolecules containing a central protein core from which a large number of glycosaminoglycan chains radiate out in a bottle brush fashion. The glycosaminoglycans are very hydrophilic and highly charged carbohydrate polymers. The high density of negatively charged groups on the glycosaminoglycan chains bound to a common core protein, gives rise to the extended conformation and the large hydrodynamic volume of the proteoglycans when in solution. The high density of fixed charges also contributes to the resistance of proteoglycans to compressive forces. Thus, as the molecular domain decreases when pressure is applied, the charge density within

the domain increases, thereby resisting compressive forces. When the pressure is released, the macromolecular domain will expand to its former volume (Hascall, 1977).

Interestingly, in the cartilage tissue the total volume which the proteoglycans occupy in the interstices of the collagen network is less than 20% of the extended form in dilute solutions (Torchia *et al.*, 1981). When the collagen fibers are damaged experimentally, the tissue takes up water and swells (Maroudas & Venn, 1977). Thus, cartilage might be regarded as an underhydrated gel of proteoglycans, where the collagen network prevents the proteoglycans from further hydration and expansion. The compact underhydrated state of the proteoglycans, prevailing even in the absence of mechanical pressure, increases their effectiveness in resisting further compression.

The mechanical properties of cartilage, then, depends on the composite properties of the collagen network and the proteoglycans entrapped within it. The collagen fibers provides the tensile strength, while the proteoglycans provides the resistance to compressive forces.

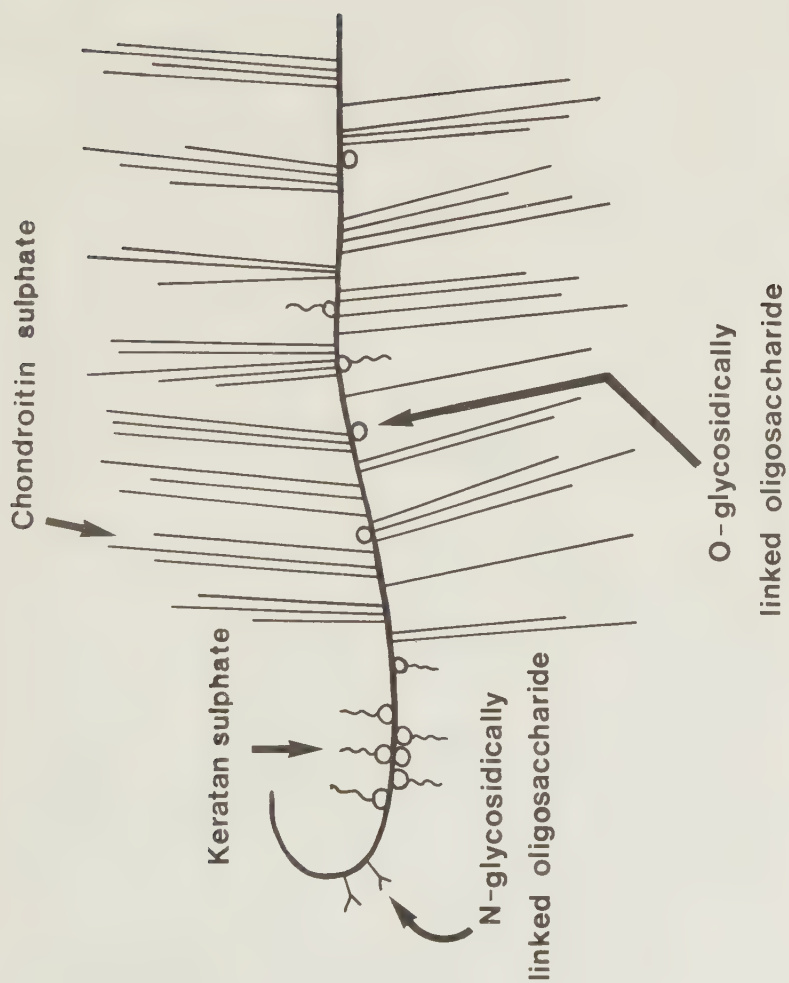
PREVIOUS INVESTIGATIONS

Structure of cartilage proteoglycans

Structural studies during the past 25 years (for references see Hascall & Heinegård, 1979; Hascall, 1981) using biochemical methods, supported by direct observation of these huge macromolecules in the electron microscope, have led to the current concept of proteoglycan structure, Fig. 1.

In the tissue the majority of the proteoglycans are present as large aggregates with a molecular weight of $50-200 \times 10^6$. Each aggregate contains 50-100 proteoglycan monomers noncovalently bound to one molecule of hyaluronic acid, as discussed below (Hardingham & Muir, 1972; Hascall & Heinegård, 1974a).

FIG.1



Most of the present knowledge of proteoglycan structure has been achieved since methods for extraction and purification of intact, undegraded proteoglycans were introduced by Sajdera & Hascall (1969). According to these methods, proteoglycan aggregates are dissociated using high concentrations of chaotropic agents, such as guanidinium chloride, in order to enable the components of the aggregate to diffuse out of the collagen network. The chaotropic agent is then removed from the extract by dialysis to "associative" conditions where aggregates are reformed (Hascall & Sajdera, 1969). The proteoglycans are purified from collagen and other non-proteoglycan proteins by equilibrium centrifugation in isopycnic CsCl gradients (Franek & Dunstone, 1967).

The proteoglycan monomers isolated from bovine nasal and tracheal cartilage have an average composition of about 87% chondroitin sulphate, 6% keratan sulphate and 7% protein (Hascall & Sajdera, 1970; Heinegård, 1972). Their molecular weights vary over the range $1-4 \times 10^6$ (Hascall & Sajdera, 1970).

According to the current model, the proteoglycan monomer consists of a central core protein with an average molecular weight of 180 000-200 000 (Hascall & Riolo, 1972) to which 80-100 chondroitin sulphate chains (Hascall & Sajdera, 1970) are covalently attached (Shatton & Schubert, 1954; Muir, 1958; Heinegård & Axelsson, 1977). Each chondroitin sulphate chain has an average molecular weight of about 20 000 and consists of a repeat disaccharide of N-acetylgalactosamine sulphate and glucuronic acid. The sequence of the linkage region between chondroitin sulphate and protein has been determined as gal-gal-xyl-serine by Rodén and coworkers (see Hascall, 1981 for references).

Another polysaccharide, keratan sulphate, is attached via an O-glycosidic bond from N-acetylgalactosamine to other serine and possibly threonine residues along the protein core (Anderson-Bray *et al.* 1967). A side chain of -gal-NANA is attached

to the same N-acetylgalactosamine residue, giving rise to the branched structure of the linkage region (Hopwood & Robinson, 1974; Kieras, 1974).

Recent investigations (Thonar & Sweet, 1979; De Luca *et al.*, 1980; Lohmander *et al.*, 1980) have shown that the cartilage proteoglycan contain two classes of oligosaccharides. The majority of mannose originally present in the proteoglycan was recovered in N-linked oligosaccharides of glycoprotein type, probably bound to asparagine residues of the hyaluronic acid binding region (Lohmander *et al.*, 1980). In addition, three different O-linked oligosaccharides bound to serine (threonine) have been discovered in proteoglycans isolated from chick limb bud or rat chondrosarcoma cultures (De Luca *et al.*, 1980; Lohmander *et al.*, 1980). One of these seems to be identical to the linkage region of keratan sulphate (Lohmander *et al.*, 1980).

The core protein contains different regions with different amino acid composition and different substitutions. Located at one end of the protein is a globular region that mediates the binding to hyaluronic acid, as discussed below. This part, accordingly termed the hyaluronic acid binding region (Heinegård & Hascall, 1974), has an apparent molecular weight of 67 000 when isolated from chondrosarcoma cultures (Faltz *et al.*, 1979). Next to the globular region is a short region to which at least 50% of the keratan sulphate is attached, the keratan sulphate enriched region (Heinegård & Axelsson, 1977). The third region is the chondroitin sulphate enriched region, where all the chondroitin sulphate chains are located together with the rest of the keratan sulphate chains (Heinegård & Axelsson, 1977). The length of this region and accordingly the number of polysaccharide chains per proteoglycan, appears to be variable (Hardingham *et al.*, 1976; Heinegård, 1977). In some molecules this region is minimally small, but in other it constitutes more than two thirds of the total protein core.

The proteoglycans in hyaline cartilage are polydisperse with regard to the variable size, and heterogenous as several subpopulations are present. Thus, 10% of the proteoglycans are not capable of aggregating (Heinegård & Hascall, 1979b). The non-aggregating proteoglycans have a chemical composition that differs from the aggregating proteoglycans. The aggregating proteoglycans can be separated into two populations with different electrophoretic mobility (McDevitt & Muir, 1971; Pearson & Mason, 1977; Stanescu *et al.*, 1977).

In addition, recent investigations have established the presence of a low molecular weight, approx. 76 300, proteoglycan in hyaline cartilage. It has a protein core with only a few chondroitin sulphate chains and no keratan sulphate attached. This type of proteoglycan does not form aggregates with hyaluronic acid (Heinegård *et al.*, 1981).

As mentioned above, most cartilage proteoglycan molecules, typically 90%, are able to interact with hyaluronic acid, an unsulphated polysaccharide with a repeat disaccharide containing glucuronic acid and N-acetylglucosamine. The hyaluronic acid binding region of the proteoglycan monomer loses its affinity for hyaluronic acid on selective modification of amino acid residues or cleavage of disulphide bonds (Hardingham *et al.*, 1976; Heinegård & Hascall, 1979a) by analogy with the active site of an enzyme. The highly specific noncovalent bond between hyaluronic acid and the hyaluronic acid binding region of the proteoglycan has a dissociation constant of about 10^{-8} (Christner *et al.*, 1978; Cleland, 1979; Nieduszyński *et al.*, 1980). The binding is further stabilized by an additional protein (Hascall & Heinegård, 1974a; Hardingham, 1979), the link protein, first described by Keiser *et al.* (1972). The dissociation constant of the ternary complex of the link-stable aggregate is not determined, but is probably even lower (Hardingham, 1979).

The hyaluronic acid binding region requires a minimal oligosaccharide of five disaccharides (a decasaccharide) for bind-

ing (Hardingham & Muir, 1973; Hascall & Heinegård, 1974b). It occupies, however, a segment corresponding to ten disaccharides (Hascall & Heinegård, 1974b). The ternary complex occupies a considerably longer portion of the hyaluronic acid, 20-25 disaccharides long (Faltz *et al.*, 1979; Kimura *et al.*, 1979; Kimura *et al.*, 1980). Thus, a decasaccharide has the minimal length required to bind strongly to the proteoglycan monomer, but is not long enough to accomodate an additional link protein.

Two forms of link proteins have been identified in bovine hyaline cartilages (Keiser *et al.*, 1972; Hascall & Heinegård, 1974a), with molecular weights of 40 500 and 46 000, respectively (Baker & Caterson, 1979b). Sometimes a third smaller link protein is present, that may be derived from the larger link proteins by proteolytic digestion (Baker & Caterson, 1979a).

Recent studies indicate that each proteoglycan monomer is stabilized by one link protein, small or large type (Heinegård & Hascall, 1974; Kimura *et al.*, 1980). Some evidence obtained (Heinegård & Hascall, 1974; Caterson & Baker, 1978) suggest that the link protein may bind to hyaluronic acid as well as to proteoglycan monomers.

Biosynthesis of the cartilage proteoglycans

The proteoglycan monomer is a very complex molecule, containing protein, polysaccharides and oligosaccharides. The newly synthesized proteoglycans are secreted from the cell into a concentrated gel of proteoglycans and collagen, where the newly formed products must be deposited at the required site. At some stage, the proteoglycan monomers are assembled into aggregates with hyaluronic acid and link protein. These facts call for a very complex sequence of processing and transport of the components that form the aggregate.

Considering the individual steps, the proteoglycan biosynthesis starts with the synthesis of the protein core on the rough endoplasmatic reticulum. The polarity of the synthesis, whether the N-terminus is located in the hyaluronic acid binding region or in the chondroitin sulphate enriched region of the polypeptide, is not known. Preliminary chemical evidence suggests that valine is the N-terminal amino acid, located in the hyaluronic acid binding region (Rosenburg *et al.*, 1979). In contrast, ^3H -serine incorporated into proteoglycans synthesized in chick limb bud cultures appears first in the chondroitin sulphate enriched region, suggesting that this region is the N-terminus (De Luca *et al.*, 1978). Little is known about the processing of the protein core that might occur after the release of the polypeptide from the ribosome, until the completed proteoglycan monomer is exported from the cell.

In contrast, many of the discrete steps and enzymes involved in the carbohydrate synthesis have been characterized (for references see Struck & Lennartz, 1980). During polypeptide synthesis or shortly thereafter (Lohmander, personal communication), the mannose rich oligosaccharides are transferred onto the core protein through the dolichol intermediate pathway. If the linkage regions of chondroitin sulphate chains and keratan sulphate chains are also synthesized at this point has not been determined. In the Golgi apparatus, the glycosaminoglycan chains are polymerized on their respective linkage regions. Finally, the sulphation of the polysaccharides occurs late in the synthesis of the proteoglycans, temporally close to their secretion.

Many questions, then, remain to be answered, concerning the biosynthesis of the proteoglycans, their secretion and subsequent organization in the collagen network. There are at least two major questions of significant biological interest concerning the biosynthesis of the proteoglycans.

The first question pertains to the above mentioned variability of the protein core (Heinegård, 1977). According to the gene-

rally accepted concept of protein synthesis, the primary structure of a protein is determined by the translated mRNA, so that all molecules of a certain protein are all identical. Proteins that are not identical, regarding their primary structure, are assumed to be translated from different mRNAs, transcribed from different genes. Some insight into these mechanisms might be provided by experiments where chondrocyte mRNA is translated in cellfree systems (Upholt *et al.*, 1980; Treadwell *et al.*, 1980). The presumptive proteoglycan protein precursor isolated, had a distinct molecular weight of about 350 000. If this is the true core protein precursor, postribosomal degradation is the most likely explanation to the variability of the chondroitin sulphate enriched region. Corroborating evidence was obtained by Kimura *et al.* (1981), using antibodies against the hyaluronic acid binding region to precipitate intracellular proteins in a guanidinium chloride extract from chondrosarcoma chondrocytes.

The other problem of general biological significance is a rather obvious one, if the size of a proteoglycan aggregate is considered. How can the cell handle and secrete a molecule with a size that approaches its own? Are the proteoglycans secreted as aggregates, or as monomers with subsequent assembly to aggregates occurring extracellularly?

To study these and related problems, methods for cell culture of bovine chondrocytes have been developed (paper I). The proteoglycan synthesized in these cultures has been characterized and compared to the proteoglycan extracted from the tissue (paper II). A novel method for separating proteoglycan aggregates from monomers, as well as estimating the size of the aggregates, has been developed (papers III and IV). Using these tools, the assembly of proteoglycan aggregates has been studied (papers V and VI).

PRESENT INVESTIGATION

Chondrocyte cell cultures (Paper I)

Biosynthetic mechanisms are preferably studied in cell cultures. Chondrocyte cultures suitable for biochemical investigations should fulfil the following requirements: 1. Large amounts of cells should be obtained. 2. The proteoglycan synthesized in cell cultures should be identical to that in the tissue. 3. The culture medium should preferably contain only chemically defined components and no supplements of biological origin, since control mechanisms (hormones etc.) are difficult to interpret, when serum proteins with unknown biological activity are present. 4. The cells used for studies of proteoglycan biosynthesis should have reached their final level of differentiation rather than going through cycles of differentiation or other alterations of the cartilage phenotype.

Most studies on proteoglycan structure have been performed using bovine cartilages. Large amounts of tissue is needed for preparation of cells, since cartilage is a rather acellular tissue. Foetal cartilages, however, have higher cell contents than adult tissues. Therefore, foetal bovine tracheal cartilage was used as source for cell preparation.

The usual procedure of collagenase digestion employed for isolation of chondrocytes (Coon & Gahn, 1966; Wiebkin & Muir, 1977; De Luca *et al.*, 1978) had to be modified in order to obtain viable cells in high yield. The newly isolated cells formed large aggregates when plated on Petri dishes. By incubating the cells for one day in a spinner flask in medium containing dextran sulphate, aggregation during subsequent culture on Petri dishes was abolished. During this incubation, the cells are allowed to recover after isolation, replacing cell surface components removed during the proteolytic digestion. In addition, traces of proteolytic enzymes contaminating the cells are efficiently removed.

Numerous reports have described alterations of the cartilage phenotype dependent on the culture conditions (Coon & Cahn, 1966; Abbott & Holtzer, 1966; Nevo *et al.*, 1972; Srivastava *et al.*, 1974; Müller *et al.*, 1977; Wiebkin & Muir, 1977; Malemud, 1978; Madsen & Lohmander, 1979). Three parameters were considered to be of primary interest: The presence of serum in the culture medium, the hydrophilic/hydrophobic character of the substrate and the cell density.

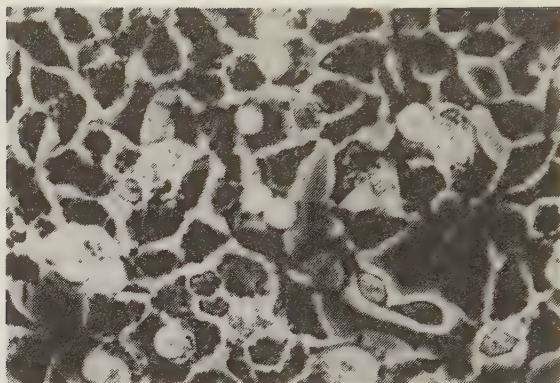
The glycosaminoglycan synthesis, measured either as ^{35}S -sulphate incorporation or accumulation of hexosamine, was rather independent of the presence of serum. In contrast, the DNA synthesis, measured as incorporation of ^3H -thymidine, was strongly dependent on the presence of serum factors.

The behaviour of the cells on different substrates was found to be dependent on the presence of serum. Thus, chondrocytes cultured in the presence of serum on hydrophilic substrates attached to the substrate and showed cytoplasmatic spreading, Fig. 2a. These monolayer cultures incorporated ^3H -thymidine at a high and constant rate during six days in culture. The chondrocytes did not attach to hydrophobic substrates in the presence of serum, but remained in suspension as single cells, Fig. 2b. These suspension cultures incorporated ^{35}S -sulphate at a higher rate but ^3H -thymidine at a lower rate than the monolayer cultures. In the absence of serum, the chondrocytes attached to either substrate but did not show any cytoplasmatic spreading, Fig. 2c. The incorporation of ^{35}S -sulphate was the same as in monolayer cultures with serum, while the incorporation of ^3H -thymidine into DNA was negligible.

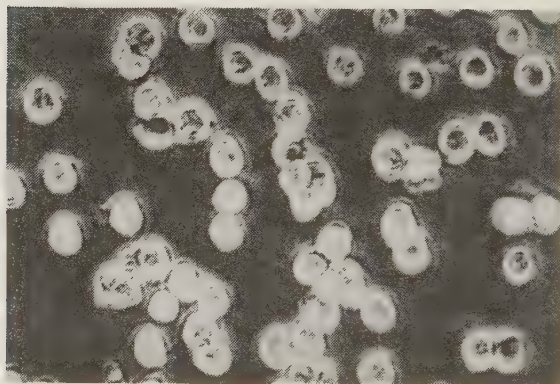
An inverse relationship was found between cell density and ^{35}S -sulphate incorporation. This is probably not caused by exhaustion of the culture medium, since the incorporation of the isotope was linear or even increased with time. The incorporation of ^3H -thymidine expectedly decreased at higher cell densities, probably because of contact inhibition.

FIG.2

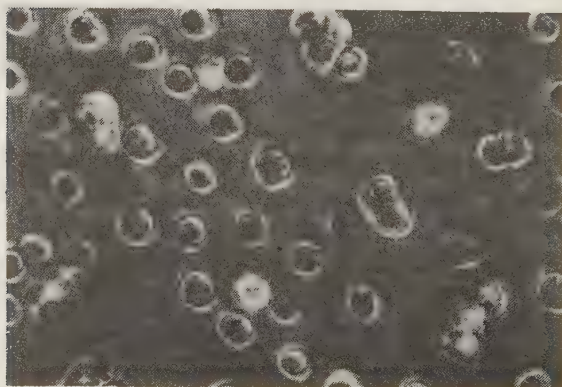
A



B



C



Extracellular glycosaminoglycans were recovered in two pools: the medium pool and the pericellular pool. The kinetics of these pools differed depending on the presence or absence of serum and the substrates used. Monolayer cultures with serum deposited 50% of the exported glycosaminoglycans in the cell layer. In culture omitting serum or in suspension cultures, only 5-10% of the glycosaminoglycans were recovered in the pericellular pool.

In a pulse chase experiment, it was found that at the end of the chase (72 hrs), only 60% of the material in the pericellular pool had been metabolized. The low turnover of the pericellular pool in this experiment corroborates the results reported by Mikuno-Tagaki and Toole (1979) on chick embryo chondrocytes pulse labelled with ^{14}C -acetate. The pericellular pool is thus unlikely to be an obligatory intermediate step for the glycosaminoglycans that are exported to the medium.

The proteoglycans secreted by the chondrocytes in the cartilage tissue are deposited in the collagen network, so that the extracellular matrix is formed. Depending on the conditions, prevailing in cell culture, matrix formation might not occur *in vitro*, even though the cells might retain their capacity for proteoglycan and collagen synthesis. Most of the proteoglycans synthesized may remain soluble in the culture medium, rather than being deposited around the cells as an insoluble matrix. A small proportion of the proteoglycans, however, may still be associated with the cells forming a pericellular pool. Since culture conditions are available with either high or low proportions of the extracellular glycosaminoglycans deposited on the cell surface, the interactions involved in forming the pericellular pool might be investigated to provide insight into factors governing cell to matrix interactions. Furthermore, the low proportion of glycosaminoglycans deposited on the cell surface facilitates investigations concerning extracellular processing, since nearly all proteoglycans exported can be accounted for in the medium.

Previously, chick embryo limb bud (Coon & Cahn, 1966; Nevo *et al.*, 1972; De Luca *et al.*, 1978), rabbit elastic cartilage (Madsen & Lohmander, 1979) or chondrosarcoma tumours (Kimura *et al.*, 1979) as cell sources. In this study, however, differentiated hyaline cartilage of mammalian origin was used as source for large scale preparation of chondrocytes. The yield of cells was about 35×10^6 cells per gram cartilage and 10 g of cartilage was obtained from one trachea.

Many investigations have shown that the synthesis of proteoglycans (Srivastava *et al.*, 1974; Madsen & Lohmander, 1979) and collagen (Müller *et al.*, 1977; von der Mark & von der Mark, 1977) depend on the environment to which the cells adapt. Thus, suspension cultures favour the production of proteoglycans while DNA synthesis is inhibited. The synthesis of cartilage specific collagen, type II, is retained in suspension cultures. In monolayer cultures, however, the proteoglycan synthesis progressively decreases and the collagen synthesis is gradually converted to type I collagen.

Characterization of proteoglycans isolated from chondrocyte cell cultures (paper II)

Proteoglycans were prepared from the medium of cell cultures, in amounts sufficient for mass analysis. The proteoglycans from cultures with or without serum, distributed in associative and dissociative CsCl density gradients like proteoglycans prepared from cartilage. The major portion of proteoglycans (94%) was recovered from the bottom third of the gradient.

The proteoglycan monomers prepared from cell cultures had similar size, measured by gel chromatography, and similar sedimentation properties, S_o -values, as the proteoglycans prepared from cartilage. The core proteins of proteoglycans derived from cell culture or cartilage had similar sizes, estimated by gel chromatography, after removal of chondroitin sulphate chains by digestion of the sample with chondroitinase ABC.

The different regions of the core protein, hyaluronic acid binding region, keratan sulphate enriched region and chondroitin sulphate enriched region could all be identified in the proteoglycans synthesized by the cultures. The hyaluronic acid binding region of the culture proteoglycans bound in link-stable aggregates was, however, degraded by trypsin to small fragments, while this region on cartilage tissue proteoglycans is resistant even to limit digestion with the enzyme. Only by reducing the enzyme to protein ratio, it was possible to obtain a component on SDS polyacrylamide gelelectrophoresis with the mobility of the hyaluronic acid binding region, although the yield was still poor. It is possible that the hyaluronic acid binding region of the culture proteoglycans have a somewhat different structure compared to the cartilage proteoglycans. It is not likely that the primary structure differs since both types of molecules are of bovine origin. Consequently any structural differences would probably be in the conformation of the proteins, perhaps due to incomplete disulphide bridge formation.

Native samples of proteoglycans, prepared from the medium, eluted in an excluded position on gel chromatography, but shifted to an included position when reduced, typical of cartilage proteoglycan aggregates. The two link proteins could be demonstrated in *in vitro* synthesized aggregates analyzed by gel electrophoresis. The size of the *in vitro* synthesized aggregates was estimated by viscosimetry and sedimentation velocity centrifugation. The fast sedimenting component from serum-free cultures had an S_0 value, close to that of aggregates prepared from tracheal cartilage after dissociative extraction (Hascall & Heinegård, 1974a). The corresponding component from serum-containing cultures had a higher S_0 value, but smaller when compared with aggregates prepared from nasal cartilage. The different S_0 values, indicating different sizes of the aggregates prepared from cultures with and without serum was supported by data from viscometry. Since the respective monomers had similar S_0 values and size, determined by gel chroma-

tography, the different sedimentation rates obtained for the aggregates probably reflects the size of the hyaluronic acid. Whether cells cultured in the presence of serum actually synthesized a larger molecular weight hyaluronic acid, or if the molecule is derived from the serum added, can presently not be answered.

Only a very small amount of ^{35}S -sulphate activity (5% of total) was found associated with the cells and released upon proteolytic digestion (paper I). In monolayer cultures of chondrocytes usually a much larger proportion of the proteoglycans is deposited in the cell layer (Lohmander *et al.*, 1976; De Luca *et al.*, 1977; Kimura *et al.*, 1979; paper I). The majority of the proteoglycans extracted from the cell layer are of cartilage type (De Luca *et al.*, 1977; Madsen & Lohmander, 1979; Kimura *et al.*, 1979) and thus might mask the presence of a minor population, specifically bound to the cell surface. Intact proteoglycans were prepared from the cell associated material by extracting the cells with guanidinium chloride. It appears from the results obtained that the small amount of proteoglycans located at the cell surface, also in this type of cultures, have the characteristics of the cartilage type proteoglycan, rather than of the cell associated heparan sulphate proteoglycan which are smaller and not aggregated to hyaluronic acid (Oldberg *et al.*, 1979). Whether this cell associated proteoglycan actually are aggregated to the hyaluronic acid at the cell surface is not known.

In conclusion, cultures of bovine chondrocytes produce proteoglycans in the presence or in the absence of serum, that are very similar to those prepared from bovine cartilages. These cultures are therefore well suited for studies of proteoglycan processing.

Zonal rate sedimentation in sucrose gradients (paper III)

Inherent to all studies of proteoglycan structure or metabolism is separation of proteoglycan aggregates and monomers as well as estimating their size. Separation may be based either on hydrodynamic volume (gel filtration) or molecular weight (centrifugation). Gel filtration on agarose gels is often employed to separate aggregates from monomers. The aggregates are excluded from the column and no information is thus obtained of the size of the aggregates. Moreover, large monomers are excluded even from the largest pore size gels, impairing the separation of aggregates and monomers. Methods based on centrifugation in an analytical ultracentrifuge requires large amounts of sample and are not applicable to radioactive samples.

Zonal velocity sedimentation in density gradients, however, provides a method with high resolution and is applicable both for analytical and preparative purposes. Sucrose is suitable for forming gradients, since the ionic composition can be varied as desired. The distribution of proteoglycans in a sucrose-gradient can, however, not be monitored by analysis of uronic acid or hexosamine, as the sucrose interferes with the analysis. Proteoglycans contain little protein. Accordingly, absorbance at 280 nm or colorimetric protein determination would not provide sensitive methods for quantitation of these molecules. In contrast, absorbance at 206 nm can be used to monitor the distribution of glycosaminoglycans, since the uronic acid and N-acetylhexosamino residues, as well as the protein, absorb light at this wavelength, unlike sucrose. Initial experiments showed that the profiles obtained by measuring the absorbance at 206 nm or by chemical analysis of protein and hexosamine (after dialysis of the fractions) are similar.

The sedimentation of proteoglycans was isokinetic with linear gradients of 10-30% sucrose. To prevent the fastest sedimenting components of the aggregates to pile up at the bottom, steeper gradients might be used. A gradient of 10-50% sucrose will slow

the sedimentation rate at the bottom two thirds of the gradient enough to prevent the largest proteoglycan aggregates to accumulate at the bottom.

A typical proteoglycan aggregate preparation is resolved into two peaks, one fast sedimenting broad peak containing the aggregates and one sharp peak, close to the top of the gradient, containing the monomers. The sedimentation of both proteoglycan aggregates and monomers varied little over the wide range of concentrations tested. As little as 19 μ g of a proteoglycan aggregate fraction was sufficient for obtaining a sedimentation profile of the sample.

The influence of salt concentration and type of salt on the sedimentation properties of protein, proteoglycan and proteoglycan fragments was studied. The sedimentation rate of albumin was not affected by the ionic environment. The sedimentation of proteoglycans and fragments thereof increased with increasing ionic strength. At low ionic strength the charged macromolecules are maximally expanded, resulting in a slower sedimentation. In all conditions proteoglycans sedimented faster than any of the fragments thereof or albumin.

A proteoglycan monomer sample was fractionated into populations of different hydrodynamic size on a Sepharose 2B column. The more retarded on the Sepharose column, the slower was the sedimentation of the sample. The result indicates that the zonal rate sedimentation fractionate according to size, which in the case of proteoglycans is closely related to molecular weight.

Proteoglycan aggregates were prepared from different cartilages. The sedimentation rate of these aggregate preparations differed, probably in accordance with the size of the hyaluronic acid.

Compared with previous applications of zonal rate sedimentation for fractionation of proteoglycans (Kato *et al.*, 1978; Karasawa *et al.*, 1979; Vasan *et al.*, 1979) the present procedure allow sensitive monitoring of the sedimentation of non-radioactive

proteoglycans. In addition, important information on the relation of proteoglycan structure to sedimentation properties is presented.

Role of link protein in formation of proteoglycan aggregates (paper IV)

The zonal rate centrifugation procedure was applied in some experiments pertaining to aggregation. First, a concentration of hyaluronic acid of 0.9% (of proteoglycan dwt) was found optimal for formation of hyaluronic acid-proteoglycan complexes, in accordance with previously published data (Hardingham & Muir, 1972; Hascall & Heinegård, 1974a).

Second, the reassociation of link stabilized proteoglycan aggregates was investigated. Samples of dissociated link-stable proteoglycan aggregates were reassociated by dialysis at different proteoglycan concentrations. The proportion of aggregates formed was surprisingly dependent on the concentration of proteoglycans during reassociation. In contrast, the proportion of aggregates obtained by mixing proteoglycan monomers with a constant proportion of hyaluronic acid was rather insensitive to the concentration of proteoglycan, as expected.

Third, the optimal proportion of link protein required to stabilize aggregates was determined. Proteoglycan monomers and link protein was mixed in varying proportions at dissociative conditions. After dialysis to associative conditions, aggregates were formed by adding 0.9% of hyaluronic acid to the samples. In order to displace any not link-stabilized monomers from the aggregates, excess hyaluronic acid oligosaccharides was added to the samples. The size and amounts of aggregates increased up to 1.5% of link protein (per proteoglycan dwt). This concentration of link protein is close to that found in tissue preparations (Heinegård & Hascall, 1974a) and corresponds to one link protein per proteoglycan monomer.

Finally, binding of link protein to proteoglycan monomer was studied. Samples of proteoglycan monomers and link protein

were mixed under dissociative conditions and dialysed to associative conditions. Zonal rate sedimentation showed that the link proteins co-sedimented with the proteoglycan monomers, indicating binding. Furthermore, incubation of the samples with isolated hyaluronic acid binding region fragments caused displacement of a large proportion of the link protein from the proteoglycan monomer. It is likely, then, that the link protein binds to the hyaluronic acid binding region of the proteoglycan monomer.

Assembly of proteoglycan aggregates (papers V, VI)

The assembly of proteoglycan aggregates in chondrocyte cultures was examined in pulse chase experiments using ^{35}S -sulphate or ^3H -leucine for labelling. Zonal rate centrifugation in sucrose gradients (10-50%) was used to separate the aggregated proteoglycans from monomers and estimate the size of the newly formed aggregates. In an initial experiment, intracellular proteoglycans extracted with guanidinium chloride were found capable of forming aggregates. Thus, the intracellular pool consists of molecules which already have the capability of interacting with hyaluronic acid, even though the binding might not occur until secreted.

The site of aggregation was studied in pulse chase experiments. At short chase times (15 min) the radioactive proteoglycans sedimented as monomers. With longer chase times, a progressively larger proportion of the radioactivity sedimented as aggregates. Consequently, the proteoglycan monomers were assembled extracellularly into aggregates. The proteoglycan aggregates became gradually larger with time, indicating that the monomers are bound to hyaluronic acid at random. With an ordered assembly, each aggregate is completed before the next is formed, and the size would not increase with time. At 3 h of chase, the size of the aggregates was similar to that obtained after six days of culture. The rate of aggregation was highest at the beginning of the chase. One explanation is that the monomers first bound to the hyaluronic acid exert a sterical hindrance to the binding of other monomers to the same aggregate.

Aggregates, link-stable as well as link-free, remain stable during sedimentation in sucrose gradients. To discriminate between the two forms of aggregates, competition experiments were performed by incubating the medium after the chase with added excess proteoglycan monomer, hyaluronic acid oligosaccharide or high molecular weight hyaluronic acid.

The newly formed aggregates were stable when incubated with exogenous proteoglycan monomer, regardless of time spent in chase, suggesting that the endogenous monomers are stabilized by link proteins when bound to the hyaluronic acid. Monomers bound into link-stable aggregates do not take part in the equilibrium with free monomers, in contrast to monomers bound into link-free aggregates (Kimura *et al.*, 1979). The proportion of endogenous monomer bound into aggregates increased, even in the presence of exogenous monomer. Apparently, the exogenous monomer did not compete on equal terms with the endogenous monomer, since the endogenous monomer was preferentially bound to the hyaluronic acid. Kimura *et al.* (1980) suggested that the link protein is bound to the proteoglycan monomer before the monomer is bound to the hyaluronic acid. The endogenous monomer is then at an advantage, and a portion of these monomer-link complexes will bind to the hyaluronic acid before the exogenous monomer compete for binding of the link protein. In contrast, no aggregates were formed when exogenous monomer competed for binding of the newly secreted link protein, indicating that the link protein bound to the proteoglycan monomer extracellularly.

The newly formed aggregates were stable when competed with hyaluronic acid oligosaccharides, again indicating that the aggregates are link-stable. The proportion of aggregates increased on incubation with oligosaccharide. This is the expected result if each monomer that becomes bound to the endogenous hyaluronic acid is immediately stabilized by link protein, while the monomer bound to the oligosaccharide still is in equilibrium with the free monomers. Consequently, an ever in-

creasing proportion of monomers will become part of link-stable aggregates, even in the presence of oligosaccharide (Kimura *et al.*, 1979).

Surprisingly, the exogenous hyaluronic acid not only competed for binding of free monomers with the endogenous hyaluronic acid, but also effectively displaced the already bound monomers at all chase times. Any effective displacement of endogenous monomers from aggregates by competition, requires an equilibrium such that bound and free monomers are continuously exchanged. Monomers bound into link-stable aggregates are not considered to take part in the equilibrium with free monomers during most experimental conditions (Hardingham & Muir, 1973; Hascall & Heinegård, 1974b; Kimura *et al.*, 1979). Depending on the binding constant and actual concentration, however, a certain proportion of the monomers bound into link-stable aggregates will detach from the hyaluronic acid, with the link protein probably still bound to the monomer. Therefore, the link-stable aggregates appear stable to competition with exogenous monomer or oligosaccharides as the proportion of monomers detached at any time is very low and the endogenous monomer-link complex is at an advantage compared to the competitor.

The exogenous hyaluronic acid, however, will compete on equal terms with the endogenous hyaluronic acid. Any free monomer-link complexes will bind to the excess exogenous hyaluronic acid, rather than to the endogenous hyaluronic acid. With time, all the endogenous monomers will accumulate on the exogenous hyaluronic acid. The different results obtained with oligosaccharides and hyaluronic acid are strong evidence for the presence of monomer-link complexes.

The binding of link protein was further investigated in experiments using ^3H -leucin for labelling the proteoglycans as well as the link proteins. Molar ratios of link protein to proteoglycan in aggregate and monomer fractions were calculated assuming that 19% of the ^3H -leucine-content is in link protein, at a molar ratio of 1:1. Direct evidence for the presence of

monomer-link complexes, anticipated from the competition experiments discussed above, was obtained. In a sample incubated for 24 hours, the molar ratio of link protein to proteoglycan was 1:1 in the aggregate fraction as well as in the monomer fraction.

When the sample was incubated with exogenous monomer, some of the link proteins were displaced from the monomer fraction (0.48 link proteins per monomer) to the aggregate fraction (2.2 link proteins per monomer). The overall ratio of link protein to endogenous proteoglycan monomer was still 1:1, indicating that the exogenous monomer had competed for binding of the link protein present in the endogenous monomer-link complexes. The exogenous monomer was bound into link-stable aggregates with the endogenous link protein as indicated by the high link protein content, relative to endogenous monomer, in the aggregate fraction. The exogenous monomer is bound to available binding sites at the hyaluronic acid, thereby increasing the size of the aggregates, rather than displacing endogenous monomers (paper V).

The presence of exogenous monomer during the chase inhibited the formation of link-stable aggregates with the ^{35}S -labelled endogenous monomer as discussed above. Since the exogenous monomer was able to compete effectively for binding of the link protein at the time of secretion, it was suggested that the link protein is secreted separately from the monomer. If so, a proportion of the newly secreted link protein would bind to the hyaluronic acid only, as the link protein has a binding site for the hyaluronic acid as well as for the proteoglycan monomer (Heinegård & Hascall, 1974; Caterson & Baker, 1978, paper IV).

In support, partially assembled aggregates, separated from the not aggregated monomers after only one hour of incubation, contained excess link protein relative to proteoglycan (2.8 link proteins per monomer). When the same sample was incubated for 16 hours, the aggregates contained the expected proportion of

one link protein per proteoglycan monomer. Probably only a minor portion of the newly secreted link proteins were bound alone to the hyaluronic acid, since each not aggregated monomer was present as complex with link in both cases.

The major route of aggregation then, is initiated by binding of the separately secreted link protein to the proteoglycan monomer. The monomer-link complex is then bound as a unit to the hyaluronic acid, Fig. 3. The resulting link-stable aggregate will gradually increase in size as additional monomer-link complexes accumulate on the hyaluronic acid. This hypothesis is in accordance with that presented by Kimura *et al.* (1980).

The properties of the aggregates assembled in cell culture were compared to those of the aggregates extracted from cartilage. The capacity of the newly synthesized proteoglycans to denature reversibly and reassociate was examined using ³⁵S-sulphate labelling. The reassociation, performed at the low concentrations of proteoglycans present in the chase medium, appear to proceed to near completion, although the actual values for aggregate content are scattered. When the reassociation was performed in the presence of exogenous proteoglycan aggregates, both the proportion and size of the reassociated aggregates was constant. Since the endogenous proteoglycans had equal capacity to form mixed aggregates regardless of time spent in culture, an extra-cellular processing resulting in improved aggregability is unlikely.

When reassociation was performed in the presence of a large excess of exogenous proteoglycan monomer, the radioactivity representing endogenous proteoglycans was recovered at the position of monomers. The exogenous monomer will compete for the binding sites on the hyaluronic acid, and only a negligible portion of the endogenous monomer will become bound. The absence of any radioactive aggregates indicates that the endogenous link protein is capable of binding also to the exogenous nasal cartilage proteoglycans monomers. If not, all the endogenous monomer should with time become bound into link-stable aggregates.

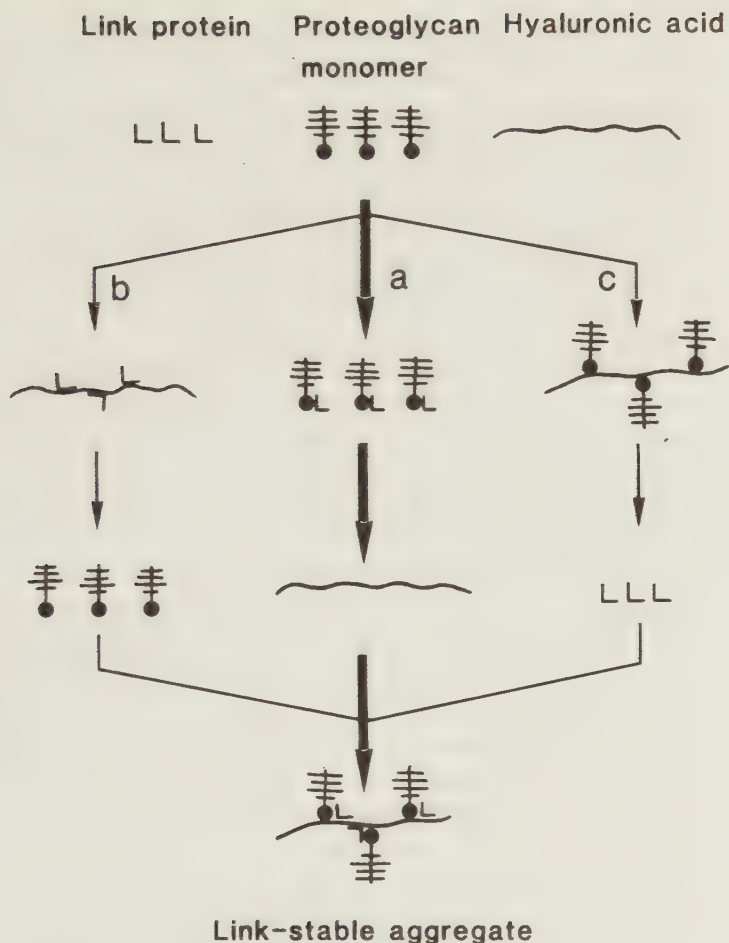


Fig.3. Possible routes of aggregation. The major route of aggregation proceeds via formation of monomer-link complexes (a). Formation of hyaluronic acid-link complexes (b) might be a minor route of aggregation. Formation of link-free aggregates (c) could not be demonstrated.

Previous work (Tang *et al.*, 1979) has shown that link-stable and link-free proteoglycan aggregates differ with regard to stability at low pH. Thus, link-free aggregates are dissociated at pH 4.0, while link-stable aggregates are stable. When the newly formed aggregates were sedimented at pH 4.0, all the radioactivity sedimented as proteoglycan monomers. Possibly, the endogenous link protein is not able to stabilize the binding between the monomer and the hyaluronic acid properly.

To test this possibility, mixed aggregates were prepared by dissociating-reassociating the endogenous proteoglycan together with bovine nasal cartilage aggregates. The resulting mixed aggregates contains endogenous monomers primarily stabilized by exogenous link protein, while the endogenous link protein is primarily bound to exogenous monomers. The reassociated proteoglycans were incubated at pH 4.0 to displace any not link-stabilized monomers. Upon zonal rate sedimentation in sucrose, all the radioactive endogenous proteoglycans sedimented as monomers. Thus, the result suggests that the low stability of the aggregates formed in tissue culture is due to differences in the hyaluronic acid binding region of the monomer rather than due to an altered link protein. This result is another indication of the difference between the cell culture proteoglycan and the proteoglycan from the cartilage tissue (paper II).

The present investigation corroborates and extends the data of Kimura *et al.* (1979; 1980). As shown by these authors the formation of link-stable aggregates in monolayer cultures of rat chondrosarcoma cells occurs extracellularly, probably via a monomer-link complex intermediate. These type of cultures differs from the type of cultures used in the present investigation, with respect to the secretion of proteoglycans into the medium. With chondrosarcoma cell cultures, only 50% of the incorporated isotope was released into the medium at short chase times (15 min), the rest being trapped in the monolayer and thus not accessible for study (Kimura *et al.*, 1979). In contrast, 90% of the incorporated activity was released into

the medium in the suspension culture of bovine chondrocytes used in the present investigation.

The separation of monomers and aggregates, by gel chromatography on Sepharose 2B, was performed in the presence of large excess of carrier proteoglycan monomer to displace not link-stabilized monomers. (Kimura *et al.*, 1979). Hence, the possibility that the monomers were secreted in link-free aggregates could not entirely be ruled out. Sedimentation in sucrose gradients gives a rapid separation between monomers and aggregates and can be performed without added carrier. The presence of only free proteoglycan monomers and no aggregates at short chase times, reported here, unequivocally shows that the aggregation with hyaluronic acid is an extracellular phenomena. Results presented by Kimura *et al.* (1980) indicate that chondrosarcoma cell synthesizes an excess of link protein relative to proteoglycan. Exogenous monomer added to the cultures bound the excess link, but was not able to compete for binding of the link protein in the endogenous monomer-link complexes. With cultures of bovine chondrocytes, no excess link protein was present. At the much higher concentration of exogenous monomer used in the present work, at least a portion of the endogenous monomer-link complexes was dissociated. None of the excess link protein present in the chondrosarcoma cultures remained bound to the hyaluronic acid only, in aggregates prepared by CsCl gradient centrifugation and subsequent gel chromatography. Possibly, the link-hyaluronic acid complexes were not stable to the conditions of preparations.

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ISOLATION AND CULTURE TECHNIQUES
OF FOETAL CALF CHONDROCYTES

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SYNOPSIS

Large quantities of differentiated mammalian chondrocytes from normal hyaline cartilage were isolated after digestion of foetal bovine tracheas with collagenase. Incubation of the newly isolated cells for 1 day in the presence of dextran sulphate inhibited formation of cell aggregates during subsequent subculture in the absence of dextran sulphate. After incubation with dextran sulphate, the cells were plated in Ham's F12 medium with or without foetal calf serum on hydrophilic or hydrophobic Petri dishes. Chondrocytes cultured on hydrophilic substrates in the presence of serum attached to the substrate and showed cytoplasmic spreading. The cells did not attach to hydrophobic substrates in the presence of serum, but remained in suspension as single cells. In the absence of serum the chondrocytes attached to either substrate, but did not show any cytoplasmic spreading. By using labelling with [35 S]sulphate and [3 H]thymidine it was shown that glycosaminoglycan synthesis did not require the presence of serum, whereas DNA synthesis required serum factors. Extracellular glycosaminoglycans were recovered in two pools: the medium pool and the pericellular pool, the latter being isolated by proteolytic digestion. The kinetics of these pools differed, depending on the presence or absence of serum and the type of substrate used. The turnover of the pericellular pool was studied in a pulse-chase experiment. At the end of the chase (72 h), only 60 % of the material in the pericellular pool had been metabolized.

INTRODUCTION

In recent years a number of details of the cartilage proteoglycan structure have been revealed (for references see Hascall & Heinegård, 1979). The progress made provides the necessary information for investigation of the different steps involved in the biosynthesis of proteoglycans.

Earlier investigations have shown that the synthesis of sulphated glycosaminoglycans by chondrocytes depends on the environment to which the cells adapt (Coon & Cahn, 1966; Abbott & Holtzer, 1966; Nevo *et al.*, 1972; Srivastava *et al.*, 1974; Müller *et al.*, 1977; Wiebkin & Muir, 1977; Malemud, 1978; Madsen & Lohmander, 1979). In the living tissue the cell is surrounded by a matrix, which is destroyed and removed during cell isolation. The relationship between the isolated cell and its microenvironment, then, is most probably disturbed. Therefore the environmental characteristics of the culture system become critical. There are three environmental parameters to consider: the culture medium, the cell density and the substrate.

Depending on the physical properties of the substrate, the cells might attach to it and eventually form a monolayer, or alternatively remain in suspension in the culture medium. Such interactions affect DNA synthesis and may also change the pattern of the cell products, *e.g.* collagen (Müller *et al.*, 1977; von der Mark & von der Mark, 1977) or proteoglycans (Srivastava *et al.*, 1974; Madsen & Lohmander, 1979) synthesized.

In the present paper a procedure for isolating tracheal chondrocytes from bovine fetuses is described. The production of sulphated glycosaminoglycans was studied in primary cultures of these chondrocytes, maintained (a) at different cell densities, (b) in medium that either contained serum or was serum-free or (c) on substrates with different adhesion properties.

MATERIALS

All chemicals used were of analytical grade. The following enzymes and chemicals were used for cell cultures: Collagenase CLS (Worthington Biochemical Corp., Freehold, N.J., U.S.A.), trypsin (type III, 2x crystallized) (Sigma Chemical Co., St. Louis, MO, U.S.A.), hyaluronidase (type I) (Sigma), papain (2 x crystallized and containing 25 mg/ml of protein) (Sigma), Ham's F12 medium (Gibco, Grand Island, NY, U.S.A.), Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] (Sigma), foetal calf serum (Flow Laboratories, Rockville Pike, MD, U.S.A.), dextran sulphate (Pharmacia Fine Chemicals, Uppsala, Sweden), carrier-free [35 S]sulphate (The Radiochemical Centre, Amersham, Bucks., U.K.), penicillin (Kabi, Stockholm, Sweden), streptomycin (Glaxo, Greenford, Middx., U.K.) and Instagel (Packard, Instrument Co., La Grange, IL, U.S.A.). Two types of Petri dishes were used: (1) hydrophobic Falcon Standard Petri dishes no. 1008 (35 mm inside diam) and no. 1007 (60 mm inside diam), and (2) hydrophilic Falcon Tissue Culture Petri dishes no. 3001 (35 mm inside diam) and no. 3003 (60 mm inside diam) (Falcon Plastics, Oxnard, CA, U.S.A.). Calf fetuses 7-9 months old were obtained from a local abattoir within 1-2 h after slaughter.

Culture media

F12 medium was Ham's F12 medium buffered with 14 mM- NaHCO_3 and containing penicillin (100 i.u./ml) and streptomycin (100 $\mu\text{g/ml}$), pH 7.4.

F12 FCS medium was F12 medium supplemented with 10 % (v/v) foetal calf serum, pH 7.4.

F12 HAD medium was F12 medium supplemented with 20 ml of 50X essential amino acids for Eagle's minimum essential medium (Flow)/1 and dextran sulphate (20 $\mu\text{g/ml}$) and buffered with 10 mM-Hepes, pH 7.4.

METHODS

Cell isolation procedure

Tracheas were removed from bovine fetuses and stripped from their mucosal layer under aseptic conditions. After removal of the perichondrium by incubation with collagenase (2 mg/ml) in 40 mM-Hepes/NaOH pH 7.4, containing 4.8 mM- CaCl_2 , dextran sulphate (20 $\mu\text{g/ml}$), penicillin (100 i.u./ml) and streptomycin (100 $\mu\text{g/ml}$) for 1 h at 37°C, the 'purified' cartilage was rinsed with F12 medium and cut into small pieces. Chondrocytes were subsequently isolated by collagenase (20 mg/g of cartilage) digestion for 10-16 h in F12 medium containing the above buffer mixture. The cells were pelleted and washed in fresh medium. Viability was tested by Trypan Blue exclusion. The cells were suspended in F12 HAD medium at a concentration of 0.5×10^6 cells/ml and transferred to a 500 ml spinner culture flask. Preincubation was then performed at 37°C for 1-2 days with continuous stirring at 60 rev./min.

Culture techniques

Cells were collected by centrifugation, washed and suspended in F12 medium or F12FCS medium. Cells were cultured at 0.5×10^6 cells/ml, corresponding to 1.0×10^5 cells/cm², unless otherwise stated. Two types of substrates were used: Falcon Standard Petri dishes (hydrophobic) or Falcon Tissue Culture Petri dishes (hydrophilic). The cultures were maintained at 37°C in a humidified atmosphere of 5 % CO_2 in air.

The cultures were harvested as follows. The medium was centrifuged at 170 g for 5 min to remove cells still in suspension. The supernatant was decanted and saved. The Petri dish was washed once with fresh medium (3 ml), the wash was centrifuged and the supernatant pooled with the previous supernatant. The cell pellet was suspended in a collagenase-containing buffer (Ham's F12 medium with 2 mg of collagenase and 2 mg of trypsin added per ml), and transferred back to the Petri dish and incubated for 30 min at 37°C on a shaker. The cell viability

after digestion exceeded 95% as judged by Trypan Blue exclusion. The cells were pelleted and washed once as described above, and the supernatants pooled. Cells, digest and medium were kept frozen until analysed. Continuous pulse experiments were performed by adding the [^{35}S]sulphate (2.5 $\mu\text{Ci/ml}$) or [^3H]thymidine (1 $\mu\text{Ci/ml}$) to the medium after the cells had been plated on Petri dishes. Pulse-chase experiments were performed as follows. Cultures were labelled for 3 days with carrier-free [^{35}S]sulphate (0.2 $\mu\text{Ci/ml}$). The chase was then initiated by adding 0.6 ml of 0.22 M- Na_2SO_4 in Fl2 medium also containing 6 μCi of [^3H]glucosamine to the 6 ml cultures. Cells were harvested as above and then incubated for 15 min at 37°C with trypsin (2 mg/ml) to solubilize cell-associated material.

Determination of synthesis of glycosaminoglycans, collagen and DNA by cells cultured in different environments

The glycosaminoglycans synthesized by the cells were quantified as the amounts of hexosamines and [^{35}S]sulphate that could be precipitated with cetylpyridinium chloride from papain digests of the fractions obtained as described above. The following procedure was used. To all samples 0.1 vol. of a papain digestion solution (0.5 M-sodium acetate/40 mM-EDTA/50 mM-cysteine hydrochloride/0.1 M- Na_2SO_4 , pH 5.0, containing 50 μl of crystalline papain/ml) was added and digestion with papain was performed at 65°C for 5 h. The glycosaminoglycans in the samples were then precipitated with 1 ml of aq. 1 % (w/v) cetylpyridinium chloride as described by Antonopoulos *et al.* (1964). The precipitates were hydrolysed in 6 M-HCl for 8 h at 100°C and samples were taken for hexosamine determinations (Antonopoulos *et al.*, 1964). Other samples were mixed with Instagel (2 ml of sample plus 3 ml of Instagel) and ^{35}S radioactivity was determined. Samples for collagen determination were freeze-dried and then hydrolysed in 6 M-HCl at 100°C for 24 h. Hydroxyproline was determined by the method of Stegeman & Stalder (1967).

DNA synthesis in the cell cultures was determined as incorporation of [^3H]thymidine. Cells were recovered, precipitated and washed with cold 5 % (w/v) trichloroacetic acid. After hydrolysis of the precipitate in 6 % trichloroacetic acid (1 ml) for 15 min at 90°C a 500 μl sample of the clear supernatant was added to Instagel (5 ml) for ^3H determination.

DNA was determined as follows. The cell pellet was lysed in 1 M-NaOH at 37°C for 1 h. DNA was precipitated and washed with cold 2 M-HClO₄ and hydrolysed in 1.5 M-HClO₄ (1.2 ml) for 30 min at 70°C. Two 500 μl samples of the clear supernatant were assayed for DNA by the method of Richards (1974).

RESULTS AND DISCUSSION

Cell isolation procedure

Tracheas were obtained from bovine foetuses during the last trimester of gestation. At this age the cartilage proteoglycans are similar to those of the adult animal (S. Inerot & D. Heinegård, unpublished work). About 10 g of cartilage was obtained from one trachea. Collagenase, at a concentration of 2 mg/ml (250-400 units/ml), was required for the liberation of cells from the matrix. Trypsin was not as efficient as collagenase and had no additional effect on the rate of cartilage digestion, when used together with collagenase. In an attempt to shorten the time of the digestion, the concentration of collagenase was raised or hyaluronidase, at two different concentrations, was added to the digestion solution (Table 1). There was no effect on the time course of the digestion, although the viability of the cells was lowered at high concentrations of either collagenase or hyaluronidase. In conclusion, we found that the cell viability was less affected by the duration of the digestion than by using higher concentrations of the enzymes. The decrease in pH during the extended digestion was kept within tolerable limits (pH 7.4-7.2) by incubating no more than 0.1 g of cartilage per ml of Earle's balanced salt solution buffered with Hepes (Shipman, 1969).

Preliminary experiments showed that the cells aggregated during the isolation procedure. Therefore dextran sulphate, which inhibits cell-substrate adhesion (Bremerskov, 1973), was tried and indeed inhibited cell aggregation completely, when used at a concentration of 20 $\mu\text{g/ml}$ in the digestion buffer.

The time required for the solubilization of the tissue could vary from 10 to 16 h, depending on the age of the foetus and the size of the cartilage pieces. Attempts to isolate cells from older bovine cartilage were not successful. Both yield (2×10^6 cells/g) and viability (50 %) of cells isolated from calf or adult tracheal cartilage were insufficient for use in primary cultures.

Preincubation

The removal of cell surface components by proteolysis during the proteolytic digestion influences cell-cell interactions and substrate adhesion. Freshly isolated chondrocytes cultured in suspension form large aggregates (Kuroda, 1964) which are difficult to dissociate. Since dextran sulphate is known to interfere with cell adhesion (Bremerskov, 1973; Kuroda, 1974) an experiment was initiated to study the effect of this polymer on aggregation of isolated chondrocytes during subsequent culture on Petri dishes in the absence of dextran sulphate. Freshly isolated cells that were kept in a spinner flask rapidly formed aggregates when removed during the first day and subcultured, whereas cells preincubated for 1 day or more in the presence of dextran sulphate remained as single cells during the subculture. No differences were observed in the quantities of glycosaminoglycans or their distribution between medium and cells with regard to the time spent in incubation with dextran sulphate. Isolated chondrocytes were always incubated in the presence of dextran sulphate for at least 1 day before being used in an experiment.

Primary culture of isolated chondrocytes

The composition of the medium, the type of the substrate and the cell density were varied as follows. (1) Cultures were

maintained either in Ham's F12 medium with no supplements or in Ham's F12 medium supplemented with 10 % (v/v) foetal calf serum. (2) Cells were plated either on hydrophilic plastic Petri dishes for use in tissue culture (hydrophilic substrate) or on standard hydrophobic Petri dishes (hydrophobic substrate). (3) Cells were plated at cell densities of 2.0×10^5 , 1.0×10^5 and 0.5×10^5 cells/cm². In all cases 0.2 ml of medium/cm² was used. Therefore the concentrations of cells were 1.0×10^6 , 0.5×10^6 and 0.25×10^6 per ml respectively.

Cultures maintained on hydrophilic substrates in F12 FCS medium showed the characteristics of traditional monolayer cultures of chondrocytes. A large proportion of the newly plated cells attached to the dish, although a number of cells remained in suspension as "primary floaters". The cells that attached to the dish often showed excessive cytoplasmatic spreading in areas with low cell density, whereas cells in areas with high cell densities remained round or polygonal. Whether this heterogeneity reflects differences in the biosynthesis of macromolecules is uncertain.

The chondrocytes in cultures maintained on hydrophobic substrates in F12 FCS medium did not attach and remained in suspension. In contrast with monolayer cultures, the chondrocytes in suspension cultures were evenly distributed and all the cells had the same rounded shape.

The cells cultured in F12 medium attached very rapidly both to hydrophilic and hydrophobic substrates in the absence of serum. The cells did not spread their cytoplasm, but remained round and appeared similar to cells cultured in suspension.

Glycosaminoglycan synthesis

Glycosaminoglycan synthesis was studied with a continuous pulse of [³⁵S]sulphate under the different culture conditions described above. The cultures were harvested at day 0, 2, 4 and 6 as described in the Methods section and the accumulation of [³⁵S]sulphate-labelled glycosaminoglycans and hexosamine was determined.

Monolayer cultures on hydrophilic substrates with Fl2 FCS medium. Extracellular glycosaminoglycan hexosamine was found in two pools with different kinetics (Fig. 1a). One pool was soluble and withdrawn with the culture medium: the medium pool. The other was closely associated with the cells and was recovered only after solubilization with proteolytic enzymes: the pericellular pool. Very small amounts of the glycosaminoglycans were associated with the cells subsequent to such treatment: the cellular pool (results not shown). The accumulation of [35 S]sulphate labelled glycosaminoglycans in the medium increased with time for 6 days (Fig. 1a). In this type of culture a fairly large proportion of the newly synthesized extracellular glycosaminoglycans (about 50 %) was retained in the cell layer, rather than secreted into the medium.

The incorporation of [35 S]sulphate per 10^6 cells in the medium pool as a function of plating cell density is shown in Fig. 2a. The increased rate of synthesis with time of culture can be explained in two ways; either the cells increased their synthesis as they adapted to the environment *in vitro*, *i.e.* by releasing factors that stimulate glycosaminoglycan synthesis (Solursh & Meier, 1973), or the number of cells increased during the culture period. The decreased rate of synthesis seen at the highest cell concentration might be due to lack of nutritional components as the medium became exhausted at the end of the culture period. The amount of [35 S]sulphate in the cellular pool (not shown in the Figure) was very low, about 1 % of the total [35 S]sulphate incorporated into polymers at day 6.

Suspension cultures on hydrophobic substrates with Fl2 FCS medium. The accumulation of the medium pool was linear with time at 0.25×10^6 and 0.5×10^6 cells/ml, then it levelled off between days 4 and 6 at 1.0×10^6 cells/ml (Figs. 1b and 2b). At a calculated cell concentration of 0.5×10^6 cells/ml the medium contained about 15 μ g of glycosaminoglycan hexosamine/ml or 1.5 μ g of glycosaminoglycan hexosamine/ μ g of DNA at day 6 (Fig. 3a). The amount of DNA increased slowly during

the culture period. Notably, the rate of synthesis of glycosaminoglycans was inversely related to the cell concentration (Fig. 2b). The kinetics of glycosaminoglycan accumulation in the pericellular pool were different from that of monolayer cultures with serum (Fig. 1b). The proportion of the extracellular glycosaminoglycans recovered in the pericellular pool was smaller (approx. 5% of the total) in this type of culture. The initial content of glycosaminoglycan hexosamine was 0.035 $\mu\text{g}/\mu\text{g}$ of DNA and increased 3-fold during the culture period. The high starting value may be due to the build-up of this pool during the preincubation performed before seeding of the cells on Petri dishes.

Serum-free monolayer cultures on hydrophobic substrates. Even in the absence of serum the cells still synthesized glycosaminoglycans although the chondrocytes had not been exposed to serum proteins at any time during the isolation procedure. The accumulation of glycosaminoglycans in the medium pool, measured as incorporation of [^{35}S]sulphate (Fig. 1c) or glycosaminoglycan content (Fig. 3b), increased linearly with time. Only at the highest cell density (1.0×10^6 cells/ml) was there a somewhat decreased rate of glycosaminoglycan synthesis between days 4 and 6. The amount of hexosamine synthesized per μg of DNA in serum-free cultures was only about 85 % of that in suspension cultures at day 6. Serum had only a moderately stimulatory effect on glycosaminoglycan synthesis. DNA initially decreased to 75 % of that at the beginning of the culture period but remained constant after day 2 (Fig. 3b). The pericellular pool (Fig. 3b) also increased linearly. The proportion of glycosaminoglycans in the pericellular pool in serum-free cultures was lower compared with that in monolayer cultures in the presence of F12 FCS medium. It is likely that less material was trapped between the single cells of the serum-free cultures than in areas with high cell density of the monolayer cultures.

Turnover of the pericellular and cellular pools

A pulse-chase experiment was initiated by adding a large excess of unlabelled sulphate rather than a complete change of medium, which might induce shedding of the cell-associated glycosaminoglycans. The efficiency of the chase was acceptable, since the amount of [^{35}S]sulphate-labelled glycosaminoglycans in the medium pool remained constant during the time period studied (Fig. 4a). The incorporation into glycosaminoglycan of [^3H]glucosamine, added during the chase, increased linearly (Figs. 4a and 4b). Therefore the chase procedure did not inhibit glycosaminoglycan synthesis. The time-dependent disappearance of [^{35}S]sulphate from the pericellular pool is shown in Fig. 4b. About 60 % of the [^{35}S]sulphate present at the beginning of the chase had been released by 72 h. The rate of turnover, even during the first 4 h, was too low to allow for a substantial portion of the glycosaminoglycans obtained in the medium pool to pass through the pericellular pool. Therefore it is less likely that the pericellular pool represents an intermediate site in the export of proteoglycans from the cells. Mikuni-Takagaki & Toole (1979) also found a low turnover of the cell-associated glycosaminoglycans in a similar experiment with chick-embryo chondrocytes pulse-labelled for 6 h with [^{14}C]acetate.

About 65 % of the intracellular [^{35}S]sulphate present at the beginning of the chase was released from the cells during 24 h (Fig. 4c). The slow release of [^{35}S]sulphate measured at the time intervals used in this experiment cannot be attributed to the turnover of the major pool of glycosaminoglycans destined for export. A considerable portion of the glycosaminoglycans for export was probably released already during the digestion with trypsin. Similar results were obtained with serum-free monolayer cultures.

Collagen synthesis

No collagen was detected in the medium in either suspension culture (with Fl2 FCS medium) or serum-free culture. The pericellular pool contained small amounts of hydroxyproline (0.25 $\mu\text{g}/\mu\text{g}$ of DNA) at the beginning of the culture period, but did not increase with time (6 days). It is possible that the pool of collagen acquired during the preincubation was maintained at the same value during the whole culture period or that the colour measured was unspecific. Since primary cultures were used, the cells might have retained a pool of ascorbic acid sufficient for a limited synthesis of collagen.

DNA synthesis

Incorporation of [^3H]thymidine by the cells was determined at days 0, 2, 4 and 6 as described above. Comparatively large differences were observed with different culture conditions (Fig. 5). Cells in culture that contained serum synthesized DNA (Figs. 5a and 5b), but a lower rate when the cell density was higher. Cultures maintained on hydrophilic substrates in the presence of serum (monolayer cultures) and with a low starting density showed a linear increase in the accumulation of [^3H]thymidine during the time studied. At high cell density the rate of incorporation was lower, and incorporation levelled off at the end of the culture period when the cells became confluent (Fig. 5a). Cells maintained on hydrophobic substrates in the presence of serum incorporated less radioactivity and reached a maximal value after 4 days in cultures at all cell densities (Fig. 5b). In serum-free cultures there was negligible synthesis of DNA at all densities (Fig. 5c). The small amount of label incorporated was probably the result of DNA repair. It appears that the DNA synthesis of chondrocytes was more susceptible to changes in the environment (e.g. the presence of serum in the culture medium) than the glycosaminoglycan synthesis.

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Table 1. Digestion of cartilage with use of different combinations of enzymes to liberate the cells

For experimental details see the text.

Expt.	Digestion solution (enzyme concentration)	Yield (10^{-6} x no. of cells/ g of cartilage)	Viability (%)
1	Collagenase (2 mg/ml)	43.3	95
2	Collagenase (2 mg/ml)	36.8	97
3	Collagenase (2 mg/ml)	20.8	94
4	Collagenase (4 mg/ml)	25.4	80
5	Collagenase (2 mg/ml) + hyaluronidase (2 mg/ml)	22.2	96
6	Collagenase (2 mg/ml) + hyaluronidase (5 mg/ml)	19.2	83



Fig. 1. Incorporation of [^{35}S]sulphate into glycosaminoglycans in different types of cultures

Experimental details are given in the text. (a) Cultures maintained in F12 FCS medium on hydrophilic substrates. (b) Cultures maintained in F12 FCS medium on hydrophobic substrates. (c) Cultures maintained in F12 medium on hydrophobic substrates.

○ Medium pool;

● Pericellular pool.

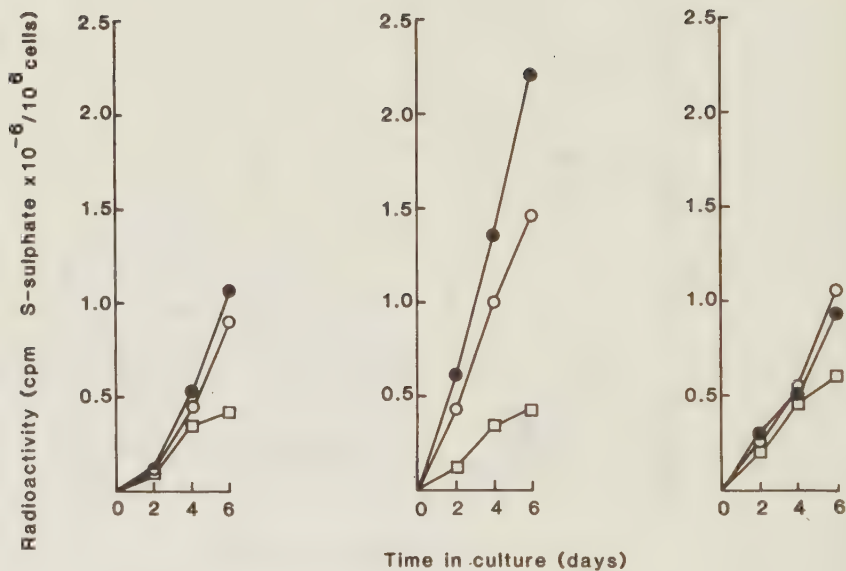


Fig. 2. Incorporation of $[^{35}\text{S}]$ sulphate into glycosaminoglycans in different types of cultures

Experimental details are given in the text. The cultures are the same as those described in Fig. 1. (a) Cultures maintained in F12 FCS medium on hydrophilic substrates.

(b) Cultures maintained in F12 FCS medium on hydrophobic substrates. (c) Cultures maintained in F12 medium on hydrophobic substrates.

□ 1.0×10^6 cells/ml; ○ 0.5×10^6 cells/ml; ● 0.25×10^6 cells/ml

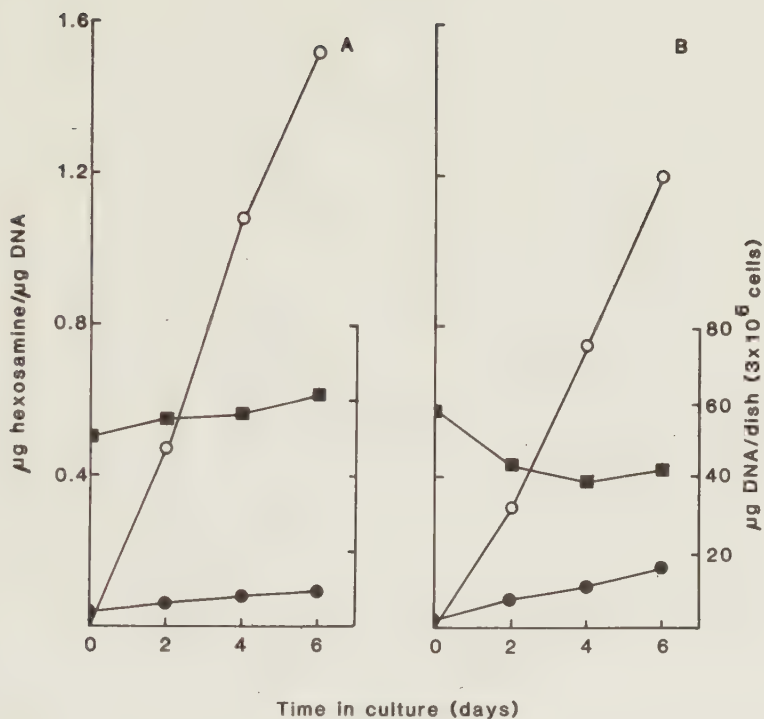


Fig. 3. Accumulation of hexosamine relative to DNA content in different types of cultures

Experimental details are given in the text. (a) Cultures maintained in F12 FCS medium on hydrophobic substrates. (b) Cultures maintained in F12 medium on hydrophobic substrates.

○ Hexosamine in medium pool; ● hexosamine in pericellular pool; ■ DNA content.

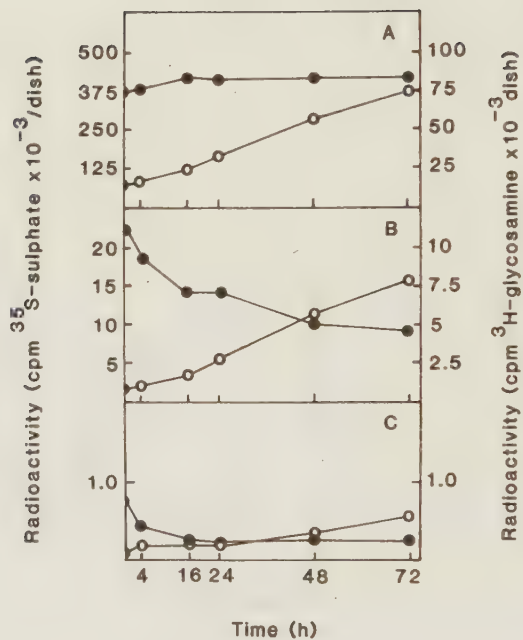


Fig. 4. Incorporation of [^{35}S]sulphate and [^3H]glucosamine into glycosaminoglycans in pulse-chase experiments. Experimental details are given in the text. (a) Medium pool. (b) Pericellular pool. (c) Cell pool.
 ● [^{35}S]sulphate; ○ [^3H]glucosamine.

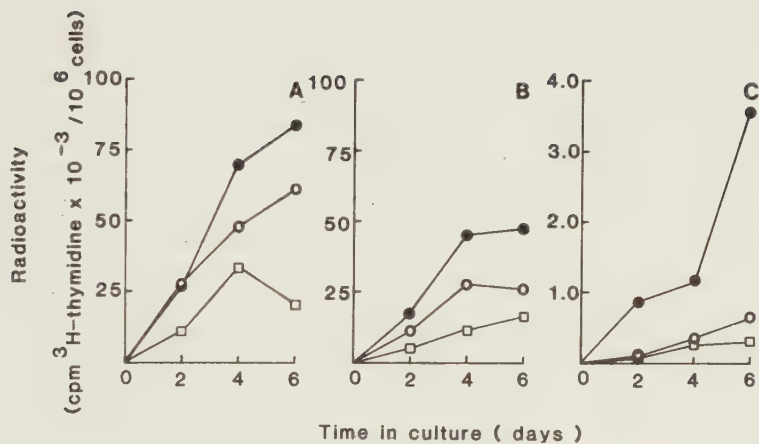


Fig. 5. Incorporation of $[^3\text{H}]$ thymidine into DNA in different types of cultures. Experimental details are given in the text. (a) Cultures maintained in F12 FCS medium on hydrophilic substrates. (b) Cultures maintained in F12 FCS medium on hydrophobic substrates. (c) Cultures maintained in F12 medium on hydrophobic substrates.

□ 2.0×10^5 cells/cm²; ○ 1.0×10^5 cells/cm²; ● 0.5×10^5 cells/cm².

FRACTIONATION AND CHARACTERIZATION OF PROTEOGLYCANS
ISOLATED FROM CHONDROCYTE CELL CULTURES

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SYNOPSIS

Chondrocyte cultures were established from foetal bovine tracheal cartilage and maintained in Ham's F12 medium with or without 10% (v/v) foetal calf serum. The proteoglycans were isolated and characterized. (1) The proteoglycans from cultures both with and without serum distributed in associative or dissociative CsCl gradients like proteoglycans from cartilage tissue. (2) The amino acid composition, protein contents and glucosamine/galactosamine ratios were grossly identical with those of the tissue derived proteoglycans. (3) Sedimentation coefficients (s^0) for the monomers were 21.0 S and 22.7 S from cultures without and with serum respectively. The s^0 values obtained for aggregates were 72.3 and 93.2 S respectively. The limiting viscosity numbers $[\eta]$ were 248 ml/g and 298 ml/g respectively. These data correspond well to those obtained for the tissue derived proteoglycans. (4) The sizes of the core proteins and chondroitin sulphate chains respectively were the same for both types of cell-culture proteoglycans and similar to those of the tissue proteoglycans. Both the keratan sulphate-rich region and the hyaluronic acid-binding region were identified. The latter, however, was not resistant to limit digestion with trypsin, in contrast with the fragment derived from the bovine nasal cartilage. (5) About 70% of the cell-culture proteoglycans chromatographed in the void volume on a Sepharose 2B column, whereas reduced and alkylated samples (monomers) chromatographed completely included in the column. The two link proteins present in A1 preparations of cartilage proteoglycans were also present in A1 preparations of cell-culture proteoglycans. (6) A minor portion (10%) of the ^{35}S -labelled proteoglycans in the cultures was associated with the cells. Reduced and alkylated samples were larger compared with the monomers in the medium and chromatographed partly (25%) excluded on the Sepharose 2B column. A larger proportion (50%) of the non-reduced samples chromatographed in the void volume of the column.

INTRODUCTION

The cartilage intercellular matrix contains two predominant macromolecular species: the collagen and the proteoglycan aggregates. The proteoglycan aggregates have a very large molecular weight of $100 \times 10^6 - 200 \times 10^6$. It has been shown (Hascall & Heinegård, 1974a,b; Heinegård & Hascall, 1974; Hardingham & Muir, 1972, 1973) that at least three different macromolecular species interact in a specific manner before cartilage proteoglycans form stable aggregates (for review see Hascall, 1977). In order to investigate the biosynthesis of the cartilage proteoglycan a procedure was developed (Björnsson & Heinegård, 1975, 1981) to isolate and culture chondrocytes from bovine cartilage, the source commonly used for isolation of proteoglycans for structural studies. Cells were obtained in quantities large enough to eliminate the need for proliferation of the cells in culture. Therefore the biosynthesis of proteoglycans can be studied with cells that represent the original population prepared from the cartilage tissue. The cells are maintained either in a medium supplemented with serum or in a chemically defined medium with no protein supplements.

In the preceding paper (Björnsson & Heinegård, 1981) only a very small amount of [^{35}S]sulphate radioactivity (5% of total) was found associated with the cells. In monolayer cultures of chondrocytes usually a much larger proportion of the proteoglycans is deposited in the cell layer (Lohmander *et al.*, 1976; De Luca *et al.*, 1977; Kimura *et al.*, 1979; Björnsson & Heinegård, 1981). Most of the proteoglycans in the medium or extracted from the cell layer are of cartilage type (De Luca *et al.*, 1977; Madsen *et al.*, 1978; Kimura *et al.*, 1979).

The present work was undertaken to characterize the proteoglycan prepared from such chondrocyte cultures maintained in a chemically defined medium with or without serum. The proteoglycans were compared with the proteoglycans extracted from bovine nasal or tracheal cartilage.

MATERIALS AND METHODS

Materials. Adult or foetal bovine tracheal cartilages were obtained from the local abattoir.

Ham's F12 medium was obtained from GIBCO (Grand Island, N.Y., U.S.A.). Guanidinium chloride (practical grade) was purchased from Sigma Chemical (St. Louis, MO, U.S.A.). All guanidinium chloride solutions were filtered through active carbon and then buffered to pH 5.8 with 50 mM-sodium acetate. Trypsin (diphenylcarbamoyl chloride-treated) and a suspension of crystalline papain (25 mg of protein/ml) was obtained from Sigma Chemical Co. Chondrotinase ABC (*Proteus vulgaris*) was purchased from Miles Laboratories (Elkhart, IN, U.S.A.) Gels for column chromatography and high-molecular-weight hyaluronic acid (Healon) were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). [³⁵S]Sulphate was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). All other chemicals were of analytical grade.

Isolation of proteoglycans from cartilage tissue. Nasal cartilage (NC) samples were extracted with 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8. Preparations of proteoglycan aggregates (NC-A1 fraction) and subsequently monomers (NC-A1-D1 fraction) were isolated as described previously (Heinegård, 1972; Hascall & Heinegård, 1974a. [The operational abbreviations previously described (Heinegård, 1972; Hascall & Heinegård, 1974a) are used throughout the text.] All preparations were extensively dialysed at 4°C against water and freeze-dried.

Isolation of proteoglycans from chondrocyte cell cultures. Cells were isolated from foetal bovine tracheal cartilage (Björnsson & Heinegård, 1975, 1981). Cultures were maintained in Ham's F12 medium that either contained 10% (v/v) foetal calf serum or was serum-free F12 medium (Björnsson & Heinegård, 1975, 1981). The medium was replaced every sixth day. After withdrawal of the medium from the Petri dishes, cells were

sedimented at $280\ g_{av}$ for 10 min. The medium was then centrifuged at $30\ 000\ g_{av}$ for 20 min to remove any remaining particulate material. The supernatant was concentrated 10-fold by using an Amicon PM-10 ultrafilter. Guanidinium chloride (7.5 M) was added to the concentrated solution to give a final concentration of 0.4 M and the pH was adjusted to 5.8. Solid CsCl was added to give a density of 1.62-1.64 g/ml. The samples were centrifuged at $100\ 000\ g$ ($r_{av}\ 6.886$) for 60 h in an MSE 8 x 25 ml angle rotor at 18°C with 17 ml of solution per tube. The tubes were emptied with the aid of a tube piercer. Contents of proteoglycans in the fractions were determined as the uronic acid contents of the material that could be precipitated from a 50 μl portion with cetylpyridinium chloride (Antonopoulos *et al.*, 1961). Proteoglycans isolated from serum-containing media are designated ChFCS-A1 fraction and those isolated from serum-free media ChF12-A1 fraction.

Subsequent dissociative centrifugation (Hascall & Heinegård, 1974a) was performed as above but in 4 M-guanidinium chloride/CsCl with a starting density of 1.52 g/ml in an MSE 8 x 25 ml angle rotor at $100\ 000\ g$ ($r_{av}\ 6.886$) for 66-72 h at 18°C .

Chemical methods. Hexuronic acid contents of samples were determined by the Bitter & Muir (1962) modification of the carbazole procedure. Column effluents were analysed for contents of uronic acid and protein by automated procedures (Heinegård, 1973). Amino acid and hexosamine contents of samples were determined after hydrolysis as described elsewhere (Heinegård, 1977). Scintillation counting of radioactivity was done with a Packard 2650 instrument, with Instagel (Packard Instrument Co., La Grange, IL, U.S.A.) as the scintillation medium.

Column chromatography. Analytical columns of Sepharose 2B (142 cm x 0.8 cm) and Sephadex G-200 (fine grade), (138 cm x 0.8 cm) were eluted at 18°C with 0.5 M-sodium acetate buffer pH 7.0. The flow rates were 4.0 and 4.2 ml/h respectively. The effluents were collected in 1.3-1.6 ml fractions.

Reduction and alkylation of proteoglycan aggregates. Aggregates were incubated for 5 h at 37°C in 4 M-guanidinium chloride/50 mM-Tris HCl buffer, pH 7.6, containing 5 mM-dithiothreitol. Subsequent alkylation was performed with 2.5-fold molar excess of iodoacetamide (Heinegård, 1977).

Viscosimetry. Viscosities of samples were measured with an Ubbelodhe dilution Viscosimeter (Cannon 100K 290) at 20.1°C. Stock solutions, with about 7 mg of material, were dialysed extensively against 0.5 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8. Dilutions of the dialysis residue with the diffusate were prepared by weight. Determinations of viscosity numbers were performed as described elsewhere (Hascall & Heinegård, 1974a).

Analytical ultracentrifugation. All sedimentation-velocity experiments were done at 20°C in an MSE Centriscan analytical ultracentrifuge, with the modified Schlieren optics. The six-place analytical rotor was used with 10 mm single-sector cells equipped with quartz windows. Apparent sedimentation coefficients were determined in 0.5 M guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8. The apparent values were extrapolated to s^0 at zero concentration by linear-regression analysis. Rotor speeds were varied from 20 000 to 40 000 rev./min, depending on the concentration of the sample. Concentrations of stock solutions and weighed dilutions were determined by the carbazole procedure (Bitter & Muir, 1962).

Digestion with chondroitinase ABC and trypsin. Fractions obtained as described above were dissolved in 0.1 M-Tris HCl/0.1 M-sodium acetate buffer, pH 7.3, and digested with 0.01 unit (nominal) of chondroitinase ABC/mg of proteoglycan for 4 h at 37°C. The samples were extensively dialysed against distilled water and freeze-dried. For a following digestion with trypsin the samples were dissolved in 1% (w/v) NH_4HCO_3 and digested with 1 μg of trypsin (diphenylcarbamoyl chloride-treated/mg of proteoglycan for 4 h at 37°C. The samples were then freeze-dried directly to remove the NH_4HCO_3 .

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Samples were electrophoresed on 8% polyacrylamide gels in the presence of 1% sodium dodecyl sulphate as described by Neville (1971). The gels were stained with Coomassie Brilliant Blue and scanned at 605 nm with a Gilford spectrophotometer equipped with a geltransporting device.

Digestion with papain. Samples (approx. 1 mg) were incubated at 50°C for 5 h with 50 µg of crystalline papain in 1 ml of 0.1 M sodium phosphate/10 mM-cysteine hydrochloride/10 mM-EDTA buffer, pH 6.5. Digests were chromatographed on the analytical Sephadex G-200 column.

Extraction of cell-associated proteoglycans with 4 M-guanidinium chloride. Cultures of chondrocytes in Fl2 medium with and without foetal calf serum were established as described elsewhere (Björnsson & Heinegård, 1975; 1981). [³⁵S]Sulphate (5 µCi/ml) was added and the cultures were left for 6 days. The culture medium was withdrawn and the cells remaining in suspension were collected by centrifugation. The Petri dishes and the cell pellets were rinsed with fresh medium and the rinse was pooled with the supernatant. The cells were suspended in 5 ml of 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8, and added back to the Petri dishes. Extraction was performed at 4°C for 24 h. The extracts were centrifuged at 105 000 g_{av} for 1 h at 4°C. The supernatants were decanted and the pellets carefully rinsed with 4 M-guanidinium chloride. The pellets were suspended in 5 ml of 4 M-guanidinium chloride and sonicated for 4 x 15 s with a Branson sonicator, and again centrifuged as above to produce an extract and a residue. Four fractions were prepared from each culture: The medium (ChFl2-M and ChFCS-M fractions), the guanidinium chloride extracts (ChFl2-Gu and ChFCS-Gu fractions), the supernatant from the sonification (ChFl2-Gu-Son and ChFCS-Gu-Son fractions) and the residues remaining after sonification (ChFl2-R and ChFCS-R fractions). Samples of all the extracts were dialysed to associative conditions and centrifuged associatively in CsCl

essentially as described above. Other samples of the extracts and of the residues were digested with papain, and the glycosaminoglycans were isolated by using precipitation with cetylpyridinium chloride essentially as described by Antonopoulos *et al.* (1961).

RESULTS AND DISCUSSION

Isolation of proteoglycans from chondrocyte cell cultures.

Proteoglycan aggregates were prepared by centrifugation in CsCl density gradients after the medium had been concentrated by ultrafiltration (Amicon PM-10). Media from the first and second weeks of culture were used with the same result. The proteoglycans isolated were not exposed to dissociating agents, and represent molecules or complexes originally assembled in the culture. The distribution of uronic acid after associative CsCl density-gradient centrifugation of ChFl2 and ChFCS fractions are shown in Figs. 1a and 1c. When cultures were pulse-labelled with [35 S]sulphate, more than 90% of the macromolecular [35 S]-sulphate of the medium was recovered from the bottom third of the associative gradient (not shown in the Figure). To avoid interference by Phenol Red from the culture medium with the carbazole procedure, a cetylpyridinium chloride precipitation step had to be introduced, as described in the Materials and Methods section. Phenol Red absorbed u.v. light, but no correction could be made since its distribution in the gradient is uneven. To isolate the proteoglycan monomer, fractions ChFl2-A1 and ChFCS-A1 were centrifuged in dissociative CsCl density gradients (Figs. 1b and 1d). The major proportion of the uronic acid (85%), representing the proteoglycan monomers, was recovered in the bottom fraction of the gradients, and the proteins distributed more to the top. The results are similar to those obtained with extracts of bovine cartilage (Heinegård, 1972).

Gel chromatography of proteoglycans on Sepharose 2B. Sepharose 2B chromatograms of fractions ChFl2-A1 and ChFCS-A1 show that 75% and 67% respectively of the uronic acid were eluted in the

void volume (Figs. 2a and 2c). In contrast, other samples that were reduced and alkylated, to abolish interaction with hyaluronic acid, chromatographed completely included, both with K_{av} of 0.24 (Figs. 2b and 2d). Similar chromatograms were obtained when bovine nasal cartilage A1 fraction was chromatographed under the same conditions (not shown in the Figure). Proteoglycans prepared from cell cultures by CsCl density-gradient centrifugation aggregate with hyaluronic acid, as do proteoglycans from cartilage (Hascall & Heinegård, 1974a).

Medium from serum-free cultures pulse-labelled with [35 S]sulphate was subjected to associative centrifugation in CsCl/0.4 M-guanidinium chloride. The gradient was divided in three fractions, A1, A2 and A3, containing 94%, 4% and 2% of the [35 S]sulphate respectively. The size of the proteoglycan monomers, as determined by Sepharose 2B chromatography (not shown) of reduced and alkylated samples, increased with density (K_{av} 0.17, 0.28 and 0.53 respectively). Large proteoglycan monomers distribute at a higher density than small monomers (Heinegård, 1977).

Cells cultured for 6 days were labelled with [35 S]sulphate 4 h before being harvested. The proteoglycan A1 fraction was isolated as described above and chromatographed on Sepharose 2B. The radioactivity representing the newly synthesized proteoglycans chromatographed identically with the uronic acid representing the total population of proteoglycan synthesized during 6 days of culture (results not shown).

The results indicate that the cells maintain their phenotype during the culture period of 6 days and that degradation of extracellular material is negligible.

Viscosimetric studies of fractions ChFl2-A1 and ChFCS-A1.

ChFl2-A1 fraction had lower viscosity numbers for all concentrations and showed a somewhat lower concentration-dependence than did ChFCS-A1 fraction (Fig. 3). The limiting viscosity numbers (η) for fractions ChFl2-A1 and ChFCS-A1 were 248 ml/g

and 298 ml/g respectively. The corresponding values for tracheal Al fraction and nasal Al fraction are 220 ml/g and 295 ml/g respectively (Hascall & Heinegård, 1974a).

The fact that ChFCS-Al fraction has a higher limiting viscosity number than that of ChFl2-Al fraction suggests that the former contains larger aggregates and/or a greater percentage of aggregates. Since both the ratio of aggregates to monomers and the size of the monomers were similar for the two preparations, as discussed below, it appears that the higher viscosity number indicates a larger molecular size of the aggregates from ChFCS fraction.

Sedimentation-velocity centrifugation of fractions ChFl2-Al and ChFCS-Al. Proteoglycan Al fractions were prepared from the medium of cultures grown in the presence of Fl2 and Fl2FCS media and analytically centrifuged at concentrations from 0.35 to 4.27 mg/ml (Table 1). The sedimentation-velocity runs were performed in 0.5 M-guanidinium chloride/50 mM sodium acetate buffer, pH 5.8, to minimize the charge effects exhibited by proteoglycans without dissociating the proteoglycan aggregates. At the highest concentration used (4 mg/ml) only one sedimenting peak was observed. At lower concentrations, however, three sedimenting peaks were observed and used for calculation of s^0 values. The apparent sedimentation coefficients obtained at the different concentrations fitted well to a straight line when plotted against their concentration, those of aggregates being markedly more concentration-dependent than those of the monomers (Table 1). The slowest-sedimenting peak (P_1) had an s^0 value of 21.0 S (ChFl2-Al fraction) or 22.7 S (ChFCS-Al fraction), correlating closely with the s^0 values obtained for proteoglycan monomers extracted from tracheal cartilage with 4 M guanidinium chloride (Hascall & Heinegård, 1974a). The fastest-sedimenting peak (P_3) had an s^0 value of 72.3 S (ChFl2-Al fraction) or 93.2 S (ChFCS-Al fraction), consistent with the viscosity data and indicating that the aggregates in ChFCS-Al fraction are larger. Between the slowest-sedimenting and the

fastest sedimenting peaks another peak sedimenting with and intermediate velocity (P_2) appeared. The behaviour of peak P_2 varied with the speed and/or the concentration. It is possible that the aggregates are not completely stable under the conditions of centrifugation and that peak P_2 represents decomposing aggregates.

Characterization of the proteoglycan monomers

Amino acid and hexosamine composition. The amino acid compositions of fractions ChFl2-Al-Dl and ChFCS-Al-Dl are nearly identical, as shown in Table 2. The results are also in good agreement with those obtained with nasal and tracheal Al-Dl fraction (Hascall & Heinegård, 1974a; Heinegård, 1972).

The galactosamine/glucosamine ratios are also similar, being 12.0 (ChFl2-Al-Dl fraction) and 12.7 (ChFCS-Al-Dl fraction) for proteoglycans from the cultures and from bovine cartilage, respectively.

Core protein. A sample of [^{35}S]sulphate-labelled ChFl2-Al-Dl fraction was mixed with 5 mg of carrier bovine nasal cartilage Al-Dl fraction and digested with chondroitinase.

Subsequent gel chromatography of the chondroitinase digests was performed on Sepharose 6B (Fig. 4a). Removal of chondroitin sulphate chains with chondroitinase ABC leaves the proteoglycan protein core still containing the keratan sulphate chains. Therefore the size distribution on gel chromatography is dependent both on the size distribution of the protein cores as well as on their degree of substitution with keratan sulphate. Part of the material was excluded from the column representing the largest size core population. Thus 39% of the radioactivity (chondrocyte culture material) and 37% of the uronic acid (tissue material), not taking the material in the total volume into account, was found in the void volume. The proportions of the total sample chromatographing in the total volume were 97.6% and 51.6% respectively. This material represents chondroitin sulphate oligosaccharide liberated by chon-

droitinase. The relative high proportion of radioactivity in the total volume may be explained if the remaining disaccharides adjacent to the link region have a lower degree of sulphation than those removed by the enzyme, as suggested by other studies (De Luca *et al.*, 1977).

Sequential digestions with chondroitinase and trypsin

Identification of the keratan sulphate-enriched region. A sample of [³⁵S]sulphate-labelled ChFl2-Al fraction was mixed with 10 mg of bovine nasal cartilage Al-Dl fraction. After sequential chondroitinase and trypsin digestion the material was chromatographed on Sepharose 6B (Fig. 4b). Three peaks were observed: two with a well-included position and one eluted later in the total volume. The position of the radioactivity peaks representing the material synthesized *in vitro* coincide with the uronic acid peaks representing the tissue material. In a previous investigation (Heinegård & Axelsson, 1977) the first peak was identified as the keratan sulphate-rich region and the second peak as fragments of the chondroitin sulphate-rich region with remaining stubs of chondroitin sulphate linkage regions and a few keratan sulphate chains. The first peak contains 48.5% of the radioactivity and 33% of the uronic acid, and the second peak contains 51.5% of the radioactivity and 67% of the uronic acid in peptide bound material. The relatively larger proportion of radioactivity in the first peak (keratan sulphate-rich region) compared with the second peak (chondroitin sulphate-rich region) may be explained by lower sulphation of the remaining chondroitin sulphate stubs of the chondroitin sulphate-rich-region fragments in the second peak as discussed above.

Identification of link protein and hyaluronic acid-binding

region. Samples of nasal cartilage fractions Al, ChFl2-Al and ChFCS-Al were separately digested either with chondroitinase only or with chondroitinase and trypsin in sequence. The digests were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in order to demonstrate the presence of link-proteins (Keiser *et al.*, 1972; Baker & Caterson, 1977)

and liberated hyaluronic acid-binding region fragments (Heinegård & Hascall, 1974). Samples digested with chondroitinase only contained two components with the mobility of link proteins (Figs. 5a-5c). Comparable amounts (about 150 μ g of proteoglycan) of the different samples were applied on the gels, but the intensity of the staining of the two link proteins derived from chondrocyte cultures was much lower than that of tissue-derived proteoglycans, indicating a lower recovery of these proteins during isolation or enzyme treatment. In all cases the larger link protein seems to be predominant. Samples digested with chondroitinase and trypsin contained one sharp peak with a high mobility corresponding to that of the small link protein and one broader peak with a lower mobility, similar to that of the hyaluronic acid-binding region fragment (Figs. 6a-6c). The samples derived from the chondrocyte cultures again showed less intense staining of both the hyaluronic acid-binding region fragment and the link protein compared with the material derived from the cartilage. The proportion between the two peaks is different in that the digest of ChFCS-Al fraction contained much less of the link protein in relation to the hyaluronic acid-binding region fragment than did the others. Whether these types of cultures actually synthesized less link protein relative to core protein or if the recovery of link protein is not as good is not known. It should be emphasized, however, that when trypsin digestion was extended the hyaluronic acid-binding-region fragments of both ChFl2-Al fraction and ChFCS-Al fraction were degraded to small peptides not capable of binding to hyaluronic acid. In contrast, the hyaluronic acid-binding region fragment of proteoglycan aggregates prepared from cartilage is resistant even to limit digestion with trypsin. The lower recovery of fragments from trypsin digests of culture proteoglycans, as discussed above, is probably due to an increased sensitivity to proteinases of their hyaluronic acid-binding region.

Digestion with papain. Single chondroitin sulphate chains were prepared from nasal cartilage fractions Al, ChFl2-Al and

ChFCS-A1 by extensive digestion with papain. The elution position on Sephacryl S-200 of the chondroitin sulphate chains as indicated by the uronic acid profiles was essentially the same for all three samples (results not shown), as would be expected from the similarity in sizes of the monomers.

Extraction of cell-associated proteoglycans with 4 M-guanidinium chloride

The distribution of radioactivity in the papain digests of the different extracts and the residues is shown in Table 3. Preliminary experiments indicated that the presence of proteinase inhibitors decreased the proportion of the cell-associated glycosaminoglycans that was extracted with 4 M-guanidinium chloride (S. Björnsson & D. Heinegård, unpublished work). Similar proportions of these glycosaminoglycans were removed by trypsin digestion (2 mg/ml, 10 min at 37°C) or extracted with 4 M-guanidinium chloride. A portion of the glycosaminoglycans not released by trypsin, however, was extractable with 4 M-guanidinium chloride (not containing proteinase inhibitors), indicating that the residues after trypsin digestion and guanidinium chloride extraction respectively differ in some respect. The major portion (90%) of the cell-associated ³⁵S label at day 6 represents the pericellular pool, removable by trypsin, rather than the intracellular pool (Björnsson & Heinegård, 1981).

The papain digests of the medium, the extracts prepared with and without sonification and the residues were chromatographed on Sephacryl S-200. In all cases the radioactivity was eluted as free chondroitin sulphate chains of similar size distribution (results not shown).

The A1-fractions of the guanidinium chloride extracts of the cells (ChF12-Gu and ChFCS-Gu fractions) were chromatographed on Sepharose 2B. About half of the radioactivity (50.0% and 52.5% respectively) was in both cases eluted with the void volume (Figs. 7a and 7c). The same samples were reduced and alkylated and chromatographed on the same column to give the

monomer size distribution (Figs. 7b and 7d). Still a proportion (25.5% of ChFl2-Gu-Al fraction and 26.6% of ChFCS-Gu-Al fraction) was eluted with the void volume, although the average size of molecules had decreased. In contrast, monomers prepared from the culture medium were included in the column, as discussed above, indicating that the monomers of the cell-associated proteoglycans were larger than those prepared from the culture medium of the same cultures. Although a proportion of the reduced and alkylated proteoglycan monomers were excluded from the column, the elution profile probably represents a continuous distribution of molecules. It appears, then, that the Al fraction of cell extracts contain monomers as well as hyaluronic acid and that aggregates are formed on dialysis of the extract.

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Table 1. Sedimentation coefficients of proteoglycan fractions
 Values were obtained at the concentrations indicated and extrapolated to zero concentration. Experimental details are given in the text. Abbreviations: GuHCl, guanidinium chloride.

Fraction	s^0 (S)		
	Peak 1	Peak 2	Peak 3
ChFl2-A1 4.27 mg/ml	4.6	-	-
2.12 mg/ml	10.9	11.9	15.1
0.70 mg/ml	19.0	36.9	54.1
0.35 mg/ml	19.8	53.6	62.4
ChFCS-A1 2.01 mg/ml	18.8	18.8	18.8
1.00 mg/ml	20.7	32.6	53.6
0.67 mg/ml	21.5	41.8	70.0
ChFl2-A1			
s^0_{20} 0.5 M-GuHCL	21.0	57.2	72.3
ChFCS-A1			
s^0_{20} 0.5 M-GuHCL	22.7	51.1	93.2

Table 2. Amino acid and hexoamine compositions of proteoglycan fractions obtained from density-gradient centrifugations in 4 M-guanidinium chloride/CsCl

Experimental details are given in the text.

Amino acid	Amino acid composition (residues/1000 residues)	
	ChFCS-Al-D1 Fraction	ChFl2-Al-D1 Fraction
Aspartic acid	69	67
Threonine	58	59
Serine	142	151
Glutamic acid	152	144
Proline	75	82
Glycine	138	146
Alanine	75	71
Cysteine	x	13
Valine	63	55
Methionine	x	x
Isoleucine	30	32
Leucine	66	70
Tyrosine	15	12
Phenylalanine	45	25
Lysine	34	16
Histidine	16	34
Arginine	23	24
Hexosamines		
$\frac{\text{Glucosamine}}{\text{Hexosamine}} \times 100$	7.9	7.7
Glucosamine/protein (w/w)	0.274	0.316
Galactosamine/protein (w/w)	3.483	3.780
Hexosamine/protein (w/w)	3.757	4.096
Protein (% of dry wt.)	7.3	7.3

^xToo low to be calculated

Table 3. Distribution of cetylpyridinium chloride-precipitable [³⁵S]sulphate-labelled material in the culture medium and guanidinium chloride extracts of cells cultured in the presence and in the absence of serum.

Experimental details are given in the text.

Fraction	Distribution (%)	
	F12 Cultures	F12FCS Cultures
Medium	96.0	92.1
Guanidinium chloride extract	2.8	6.9
Guanidinium chloride sonicate	1.1	1.0
Residue	0.04	0.02

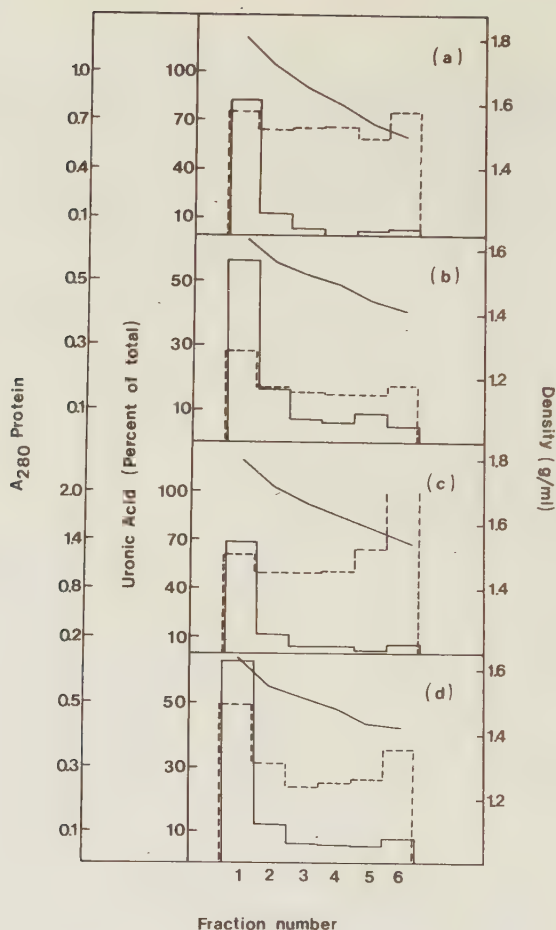


Fig. 1. Distribution of uronic acid and protein in the centrifuge tube from density-gradient centrifugations of proteoglycan fractions

Experimental details are given in the text. (a) ChFl2 medium centrifuged for 66 h in 0.4 M-guanidinium chloride/CsCl, pH 5.8, with a starting density of 1.62 g/ml. (b) ChFl2-Al fraction centrifuged for 66 h in 4 M-guanidinium chloride/CsCl, pH 5.8, with a starting density of 1.52 g/ml. (c) ChFCS medium centrifuged for 66 h in 0.4 M-guanidinium chloride/CsCl, pH 5.8, with a starting density of 1.62 g/ml. (d) ChFCS-Al fraction centrifuged for 66 h in 4 M-guanidinium chloride/CsCl, pH 5.8, with a starting density of 1.52 g/ml.

— Uronic acid; - - - protein; — density

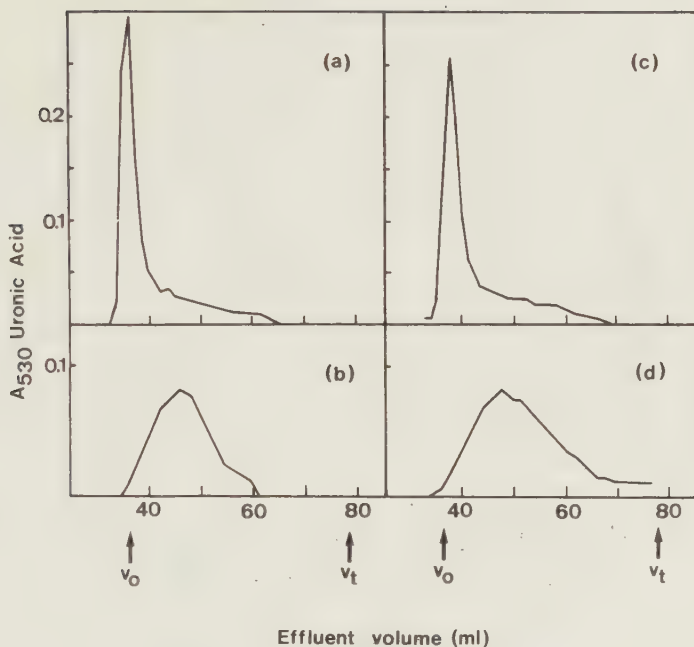


Fig. 2. Gel chromatography of proteoglycan fractions on a Sepharose 2B column

Experimental details are given in the text. Elution was with 0.5 M-sodium acetate buffer, pH 7.0; 1.26 ml fractions were collected and analysed for uronic acid. (a) ChF12-Al fraction. (b) ChF12-Al fraction, reduced and alkylated. (c) ChFCS-Al fraction. (d) ChFCS-Al fraction, reduced and alkylated.

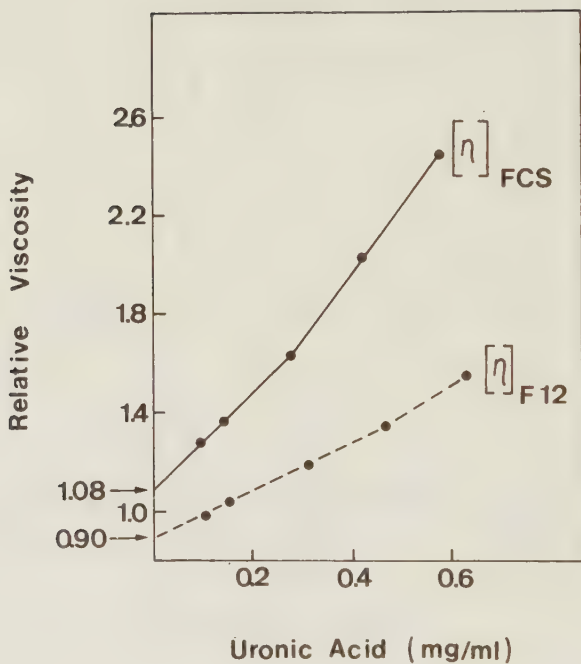


Fig. 3. Viscosity of proteoglycan fractions at different concentrations in 0.5 M-guanidinium chloride

Experimental details are given in the text. Arrows indicate the limit viscosity numbers obtained.

—, ChFCS-Al fraction; ----, ChF12-Al fraction.

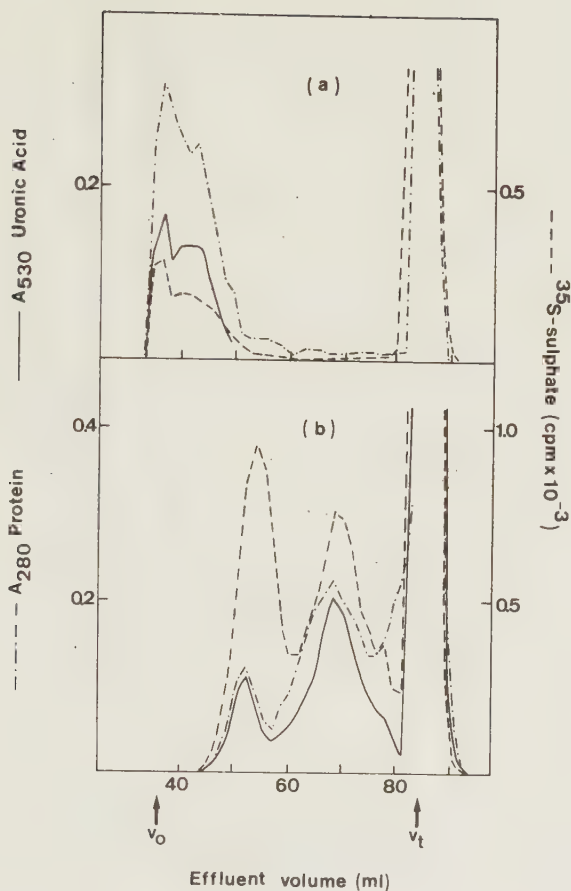


Fig. 4. Gel chromatography of enzymically digested proteoglycan fractions on a Sepharose 6B column

Experimental details are given in the text. Elution was with 0.5 M-sodium acetate buffer, pH 7.0; 1.6 ml fractions were collected. (a) ChFl2-A1 fractions digested with chondroitinase (b) ChFl2-A1 fraction sequentially digested with chondroitinase and trypsin.

—, [35 S]Sulphate radioactivity; ----, uronic acid; -.-.-, protein.

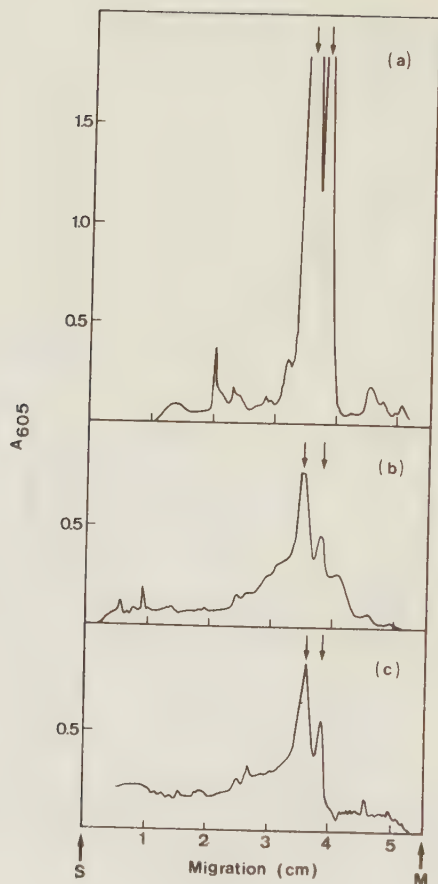


Fig. 5. Densitometric scans of sodium dodecyl sulphate polyacrylamide gels electrophoretograms of enzymically digested proteoglycan fractions
Experimental details are given in the text.
S, starting point; M, position of the tracking dye. (a) NC-Al digested with chondroitinase. (b) ChFl2-Al fraction digested with chondroitinase. (c) ChFCS-Al fraction digested with chondroitinase. Arrows indicate the positions of the link proteins.

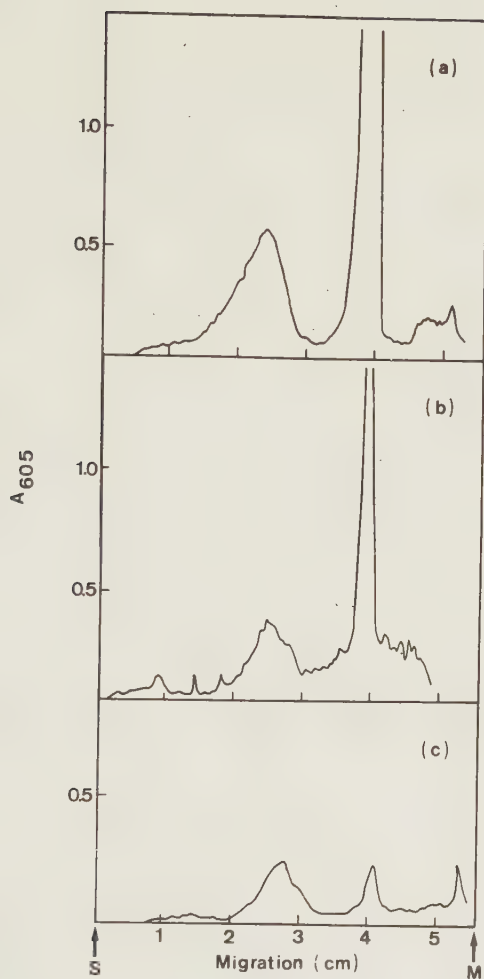


Fig. 6. Densitometric scans of sodium dodecyl sulphate/polyacrylamide gel electrophoretograms of enzymically digested proteoglycan fractions. Experimental details are given in the text. S, starting point; M, position of the tracking dye. (a) NC-A1 fraction digested with chondroitinase and trypsin. (b) ChF12-A1 fraction digested with chondroitinase and trypsin. (c) ChFCS-A1 fraction digested with chondroitinase and trypsin.

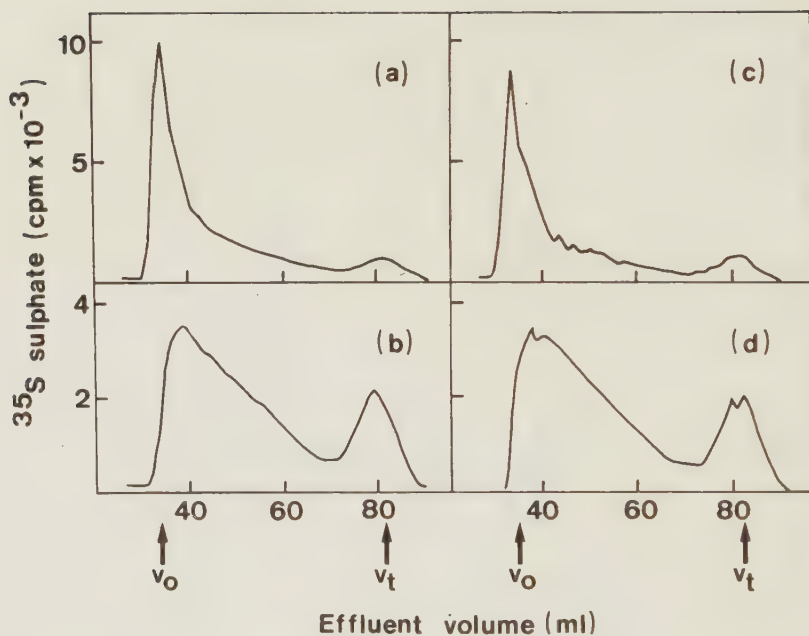


Fig. 7. Gel chromatography of reduced and alkylated proteoglycan fractions on Sepharose 2B column

Experimental details are given in the text. Elution was with 0.5 M-sodium acetate buffer, pH 7.0; 1.26 ml fractions were collected and analysed for radioactivity.

(a) ChFl2Gu-Al fraction. (b) ChFl2Gu-Al fraction, reduced and alkylated. (c) ChFCSGu-Al fraction. (d) ChFCSGu-Al fraction, reduced and alkylated.



ZONAL RATE CENTRIFUGATION OF PROTEOGLYCANS IN
SUCROSE GRADIENT

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ABSTRACT

Sensitive detection of proteoglycan and protein in concentrated sucrose solutions was obtained by measuring the absorbance at 206 nm. This method for detection has been used to study conditions affecting zonal rate sedimentation of proteoglycans and proteins in sucrose gradients. The ionic environment in the gradients markedly affects the sedimentation rate of the polyanionic proteoglycans and fragments thereof, while proteins sediment at an approximately constant rate irrespective of the ionic strength. The procedure could be used to separate proteoglycan aggregates from monomers and to determine size distributions of the components. In separate experiments, intact and fragmented proteoglycans were separated from proteins.

INTRODUCTION

Proteoglycans are large macromolecules containing a central protein core with a number of negatively charged polysaccharide side chains radiating out in a coomblike fashion (Hascall & Sajdera, 1970; Rosenberg *et al.*, 1975; Thyberg *et al.*, 1975). Their large hydrodynamic size and their large number of negatively charged group hamper their fractionation according to size by gel chromatography or according to charge by ion exchange chromatography. To date, however, some data on size distributions have been gathered by analytical ultracentrifugation (Hascall & Sajdera, 1970) and by gel chromatography on low percentage agarose gels (Heinegård, 1977). Some proteoglycans, in particular from cartilage, form very large complexes. Several proteoglycan monomers can bind noncovalently to one hyaluronic acid molecule (Hardingham & Muir, 1972; Hascall & Heinegård, 1974). The proteoglycan aggregate formed has a molecular weight of $100 \cdot 10^6$ or more. Most fractionation techniques do not allow fractionation of such large particles. The only tool suited for studies of the size distribution of proteoglycan aggregates has been the analytical ultracentrifuge.

Because of the problems associated with fractionating proteoglycans, much interest have been devoted to alternative techniques. An elegant micromethodology to determine S-values of aggregates and monomers has been developed by Pita *et al.*, 1979, using sedimentation in CsCl-gradients. The rather tedious procedure to prepare and analyze these gradients, however, make them unsuitable for analyses of large numbers of samples. Radioactively labelled proteoglycans from chondrocyte or limb bud cell cultures have been fractionated by zonal rate centrifugation in gradients of sucrose or glycerol to yield 2-3 populations of proteoglycan monomers (Kato *et al.*, 1978; Vasan & Lash, 1979; Karasawa *et al.*, 1979). In other studies purified proteoglycans have been fractionated into aggregate and monomer by zonal rate centrifugation in NaCl gradients (Sherblom & Cleland, personal communication; Hoffman,

1979). The implications of these investigations are limited as the sedimentation behaviour of monomers and aggregates in these gradients has not been characterized.

The advantages of zonal rate centrifugation are several. It is possible to fractionate even the largest molecular size material. As compared to chromatographic procedures the absence of a matrix, minimizes losses due to unspecific adsorption, a feature essential for analysis of small amounts of sample.

The aim of the present investigation was to develop a sensitive procedure to fractionate and detect proteoglycans and other macromolecules in sucrose gradients. Sucrose was the gradient forming medium of choice, as the ionic environment during centrifugation can be varied within wide limits. Alternative gradients media could be glycerol, methrizamide and salts like cesium chloride and sulphate.

MATERIALS AND METHODS

Bovine articular, nasal and tracheal cartilages were obtained from the abattoir within minutes after the death of the animal. Cartilage was dissected free from surrounding tissue, sliced into small pieces and stored frozen at -60°C until extraction.

Extraction with 4 M guanidinium chloride and subsequent preparation of proteoglycan aggregate (A1) and monomer (A1-D1) fractions by CsCl density gradient centrifugation was done as described elsewhere (Heinegård & Axelsson, 1977; Heinegård & Hascall, 1979).

A sample (40 mg) of bovine nasal proteoglycan monomers were subfractionated according to size by chromatography on a Sepharose CL2B column (2.5 x 145 cm) eluted with 4 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8. The effluent material was pooled to yield 8 subfractions.

Chondroitin sulphate peptides (clusters) were the very same, that were prepared by trypsin-chymotrypsin digestion of proteoglycans, separated by chromatography on Sepharose 6B and then extensively characterized as described elsewhere (Heinegård & Hascall, 1974). Chondroitin sulphate chains were prepared from bovine nasal cartilage by papain digestion and precipitation with cetylpyridinium chloride (Antonopoulos *et al.*, 1964).

Samples to be centrifuged were dissolved in the appropriate buffer (usually 0.15 M sodium chloride, 0.005 M sodium phosphate, pH 7.0) at the required concentration and slowly stirred in the cold for 12 h.

Chemical methods

Protein content of fractions was determined using the Lowry procedure (Lowry *et al.*, 1951) and hexosamine contents were determined with a modification of the Elson and Morgan procedure (Antonopoulos *et al.*, 1964). All chemicals used were of analytical grade. Sepharose and Sephadex gels were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

Preparation of sucrose gradients

Solutions of 10, 30 and 50% sucrose containing the required salt and buffer were prepared. Usually 0.50 M sodium chloride, 5 mM sodium phosphate, pH 7.0, was used. All solutions were filtered through a Millipore type AA filter (0.80 μ m) to remove particles. Monitoring the absorbance of the gradients at 206 nm required the background absorbance of the solutions to be adjusted to the same value. The absorbance at 206 nm (at 0.05 or 0.02 absorbance unit full scale deflection) was recorded while the most concentrated solution was continuously pumped through an Uvicord S (LKB, Sweden) flow-through-photometer. The absorbance of the less concentrated solution could then be adjusted to the same value by addition of a few drops of an aqueous solution of a compound with a high absorbance at 206 nm, while the solution was recirculated through the Uvicord. Usually 0.2% (w/v) sodium azide or 8 M urea was used.

The use of a peristaltic pump giving negligible pulses was found important for achieving stable baseline and a high sensitivity. In our experiments an LKB Multiperpex pump was used. The gradients were prepared by using two peristaltic pumps and a mixing chamber with a rotating magnetic bar. The sample solution was layered on top of the gradient by using a Hamilton gastight syringe. The gradients usually had a volume of 3.6 ml with 100 μ l of sample in MSE 6 x 4.2 polycarbonate tubes (70 mm length). The MSE Centriscan centrifuge was used with a 6 x 4.2 ml titanium swing out rotor. Centrifugation was done at 50,000 rpm (240,000 g; r_{av} 8.802 cm) and a temperature of 20°C. The centrifuge was operated with the brake on during deceleration. In other experiments using conventional preparative ultracentrifuges it has been found better not to use the brake.

After centrifugation the tubes were emptied. A capillary was carefully pushed through the gradient to rest at the bottom of the tube. The capillary was connected by teflon tubing to the Uvicord S, which in turn was connected by teflon tubing to a Multiperpex pump fitted with the 1 mm i.d. pump tubing. The pump was connected to an LKB Redirac fraction collector. It is essential that the entire system is filled with the most concentrated sucrose solution before use, since the gradient has to be pumped from the top of the Uvicord flow cell rather than from the bottom as is usually recommended. It is also important to remove any air bubbles in the system before the gradient is emptied. The particular pump used is equipped with a stepmotor which can regulate the fraction collector to change after a defined volume has been pumped. Fractions were collected using this feature, mainly to indicate the volume of the gradient emptied at each moment. Usually a setting of the Uvicord of 0.2 A, full scale deflection, was used.

RESULTS AND DISCUSSION

Analysis of the sucrose gradients

In an initial experiment, nasal cartilage proteoglycan A1-fraction was fractionated to yield aggregate and monomer. After centrifugation for 36 min in a 10-50% sucrose gradient, the tube was emptied and the effluent was continuously monitored for absorbance at 206 nm using the Uvicord S. The fractions collected were extensively dialysed against water and freeze-dried. They were then redissolved in distilled water and analyzed for contents of protein. Aliquots were withdrawn, hydrolysed and hexosamine contents were determined. The profiles for UV-absorbance, protein and hexosamine closely follow each other, Fig. 1, indicating that either measure can equally well be used to monitor for proteoglycan distribution. Indeed, at 206 nm proteins have a very strong absorbance. At this wavelength the N-acetamido and carboxyl groups of the glycosaminoglycans will provide much higher absorbance than simple sugars like sucrose. In a separate experiment a protein (bovine serum albumin), on a weight basis, was found to give 6 times the absorbance of protein free chondroitin sulphate. The absorbance value recorded for a cartilage proteoglycan, then, is approximately half due to protein and half due to glycosaminoglycans. The profiles in Fig. 1 show a very broad faster sedimenting peak containing the proteoglycan aggregates and a slower sedimenting peak, remaining near the top of the tube, containing the proteoglycan monomers. The aggregates appear polydisperse and contain some extremely fast sedimenting material. The proportion of aggregate to monomer was about 85:15 in accordance with previous data (Hascall & Heinegård, 1974).

Characteristics of the sedimentation

Dependence on the concentration of sucrose. The sedimentation distance of proteoglycan monomers as a function of time was determined for 10-30% and 10-50% (w/w) linear sucrose gradients, Fig. 2A. Expectedly the sedimentation was slower in 10-50% sucrose gradients and was linear only for 2-3 h of centrifuga-

tion, corresponding to 30-35% of the tube length. At this time the sedimentation rate decreased. In contrast, sedimentation in 10-30% sucrose gradients was linear with time for at least 5 h, corresponding to about 80% of the tube length. The 10-30% sucrose gradients appear isokinetic and should be preferred for most applications. To prevent the fastest sedimenting proteoglycan aggregates to accumulate at the bottom of the tube the 10-50% sucrose gradients were used, when aggregate containing samples were centrifuged.

Dependence on the concentration of proteoglycan. The concentration dependence of sedimentation of proteoglycan aggregates and monomers in proteoglycan Al-fraction was studied, Fig. 2B. Centrifugation was for either 36 or 60 min in 10-50% sucrose gradients. By using a full scale deflection of 0.05 A unit of the Uvicord it was possible to analyse the Al-fraction at 0.19 mg/ml (19 μ g/sample). Notably, the concentration dependence was very small for the sedimentation of both aggregate and monomer at the wide range of concentrations monitored, Fig. 2B. Therefore, the procedure should be useful also for studying samples where the exact concentration of proteoglycan is not known.

Dependence on ionic environment. The effect of salt concentration and type of salt on the sedimentation properties of protein, proteoglycan and fragments thereof was studied. Albumin was chosen as a model protein. Nasal cartilage proteoglycan monomers (Al-D1) were used for reference. Proteoglycan trypsin and trypsin-chymotrypsin fragments, which represent chondroitin sulphate peptide clusters (Heinegård & Hascall, 1974), were centrifuged as well as hyaluronic acid and isolated chondroitin sulphate chains. Consequently, heavily charged (polyanionic) sulphate macromolecules of large, intermediate and small size (proteoglycans, chondroitin sulphate peptides and chains, respectively), less charged large macromolecules (hyaluronic acid) containing no sulphate groups and little charged molecules (albumin) were sedimented in the sucrose gradients. Three types of centrifugation conditions with regard to ionic strength were tried. Centrifugation was done at low ionic strength (0.005 M

sodium phosphate) and in two different salt environments at intermediate ionic strength (0.5 M sodium chloride and 0.5 M CsCl, respectively, both in 0.005 M sodium phosphate). In each case 300 µg of sample was centrifuged. Centrifugation times had to be varied due to the marked influence of the salt on the sedimentation properties of the polyanions.

In all conditions proteoglycans sedimented faster than any of the fragments thereof or albumin, Fig. 3. Trypsin fragments expectedly sedimented faster than the smaller trypsin-chymotrypsin fragments and chondroitin sulphate chains, Fig. 3. The relative sedimentation rate of proteoglycan, chondroitin sulphate peptides, chondroitin sulphate chains and hyaluronic acid was similar in all conditions of centrifugation. The absolute sedimentation, however, was fastest in cesium chloride, intermediate in sodium chloride and markedly slow at low salt concentration. In contrast the absolute sedimentation rate of albumin was rather constant in the different ionic environments, Fig. 3. The experiments show that by altering the ionic environment in the gradient it is possible to change the sedimentation rate of polyanionic proteoglycans and chondroitin sulphate peptides. It is important to note that polyanionic macromolecules in contrast to proteins are expanded at low ionic strength and therefore sediment relatively slower. At higher ionic strength the polyanionic macromolecules are more compact and therefore sediment faster. In gradients containing 0.5 M cesium chloride, good separation of intact proteoglycan monomer and albumin was achieved. By using divalent ions or compounds interacting more strongly with the sulphate groups or with particular populations of proteoglycans, it is also possible to improve the separation of proteoglycan classes containing different glycosaminoglycan side chains. Indeed, in separate experiments it was shown that calcium ions may affect the sedimentation properties of proteoglycans (data not shown).

Size of proteoglycan monomer. The fractionation of proteoglycans according to size was studied using both 10-50 and 10-30% sucrose gradients. A sample of proteoglycan monomer

was subfractionated according to size by gel chromatography on Sepharose Cl-2B, Fig. 4. Three of the subfractions obtained were centrifuged in the sucrose gradients for 3 h (at 240,000 g_{av}), Fig. 4. The more retarded on the Sepharose column, the lower was the sedimentation rate of the sample, indicating that the separation system indeed does fractionate according to size, which in the case of proteoglycans is related to molecular weight.

Size of aggregate. Bovine tracheal cartilage proteoglycan aggregates have a lower S-value and are smaller compared with those from bovine nasal cartilage (Hascall & Heinegård, 1974). They were found to differ accordingly, with regard to their sedimentation behaviour in the sucrose gradients, Fig. 5. The aggregates from bovine articular cartilage, sedimented even slower, Fig. 5. The monomers from tracheal and nasal cartilage, however, sedimented with approximately the same rate, Fig. 5, corroborating previous data (Hascall & Heinegård, 1974). The results obtained show that zonal rate sedimentation in sucrose gradients can be used to monitor size of aggregates as well as ratio of proteoglycan aggregate to monomer.

GENERAL DISCUSSION

Zonal rate centrifugation in sucrose gradients has been shown to be an extremely useful tool in fractionating proteoglycans. The important advantages include the absence of matrix, no upper limit on size determinations, ease of running multiple samples, speed of assay and a much improved sensitivity. As little as 20 μg of proteoglycan suffices for studying proportion of aggregate and monomer and their respective size distribution.

ACKNOWLEDGEMENT

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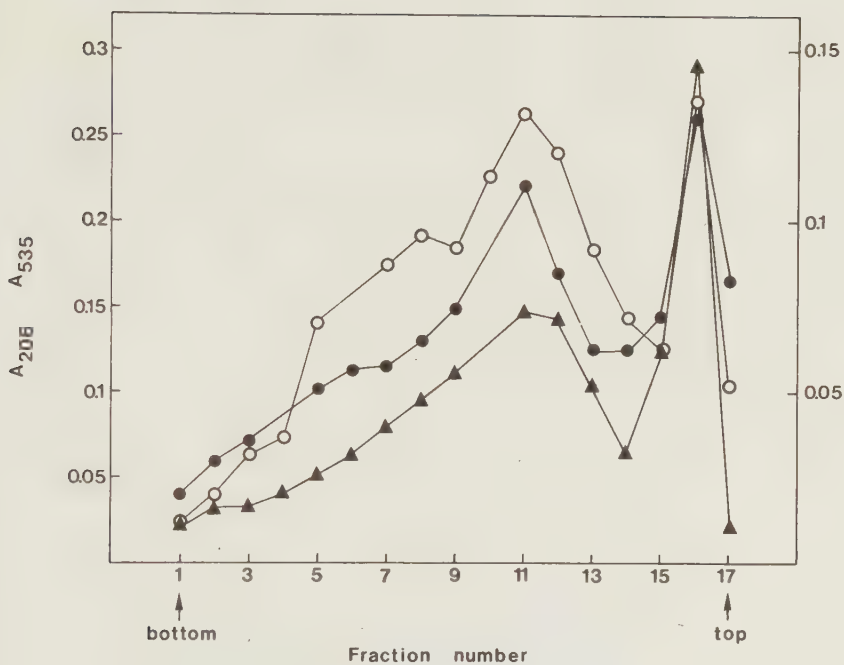


Fig.1. Zonal rate sedimentation of nasal cartilage A1-fraction in 10-50% sucrose. Experimental details are given in the text. Analysis for absorbance at 206 nm (▲), hexosamine (○) and protein (●).

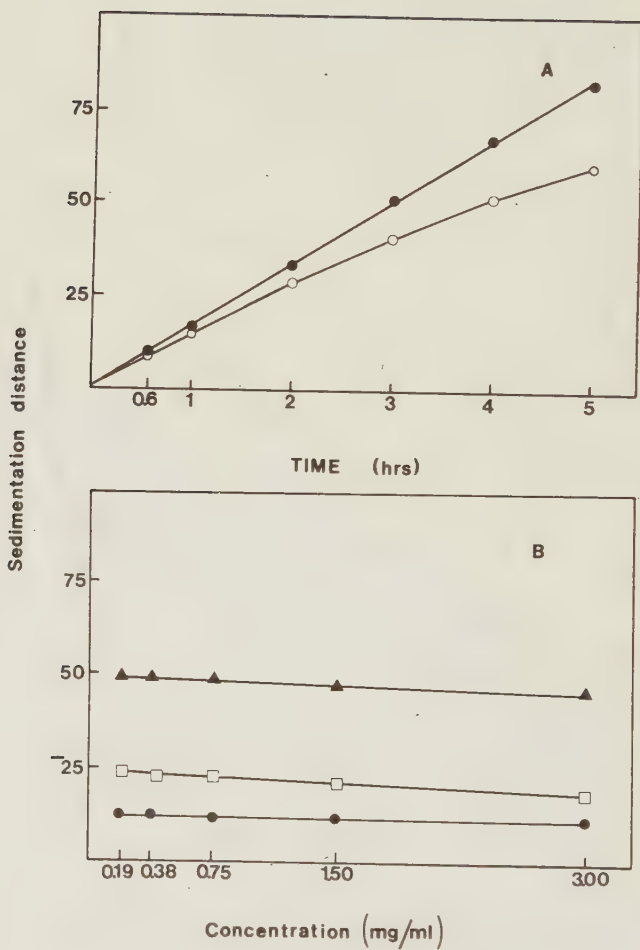


Fig.2A. Sedimentation dependence on duration of centrifugation. Experimental details are given in the text. 10-30% sucrose (●), 10-50% sucrose (○). Sedimentation expressed as distance sedimented over distance meniscus to bottom of tube.

Fig.2B. Concentration dependence of sedimentation rate in 10-50% sucrose gradients. Experimental details are given in the text. Aggregates (▲) and monomers (●) centrifuged for 36 min. Monomers (□) centrifuged for 60 min.

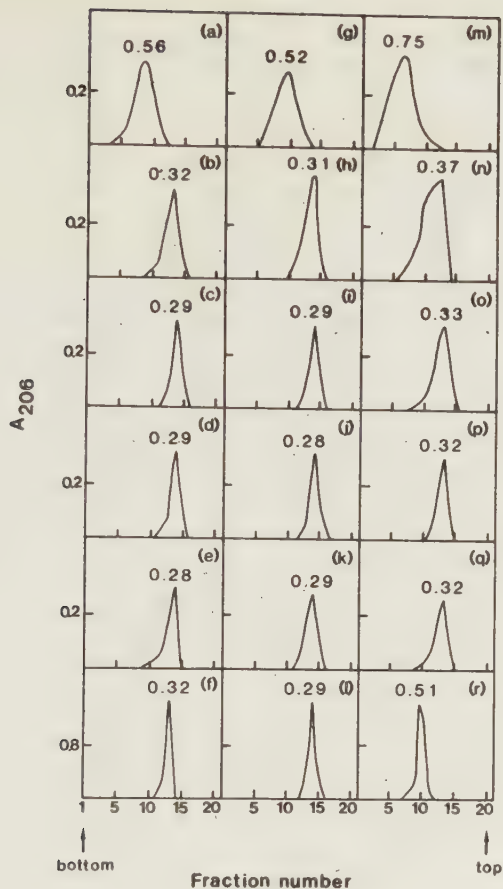


Fig.3. Sedimentation dependence on ionic environment.

Sedimentation of proteoglycan monomers (a,g,m), chondroitin sulphate peptides liberated from proteoglycan monomers by trypsin digestion (b,h,n), chondroitin sulphate peptides liberated from proteoglycan monomers by sequential digestion with trypsin-chymotrypsin (c,i,o), chondroitin sulphate chains isolated from proteoglycan monomers (d,i,p), high molecular weight hyaluronic acid (Healon^R) (e,k,q) and albumin (f,l,r) in various ionic conditions. Centrifugation in 10-50% sucrose containing 0.5 M-sodium chloride, 5mM-sodium phosphate, pH 7.0 (a-f), 0.5 M-cesium chloride, 5 mM-sodium phosphate, pH 7.0 (g-l) and in 5 mM-sodium phosphate, pH 7.0 (m-r). Samples were dissolved in 0.15 M-sodium chloride, 5 mM-sodium phosphate, pH 7.0 (a-f), 0.15 M-cesium chloride, 5 mM-sodium phosphate, pH 7.0 (g-l) and 5 mM-sodium phosphate, pH 7.0 (m-r). Centrifugation for 3 h (a-f), 1.5 h (g-l) and 10 h (m-r). The numbers express the distance sedimented over distance meniscus to bottom of the tube.

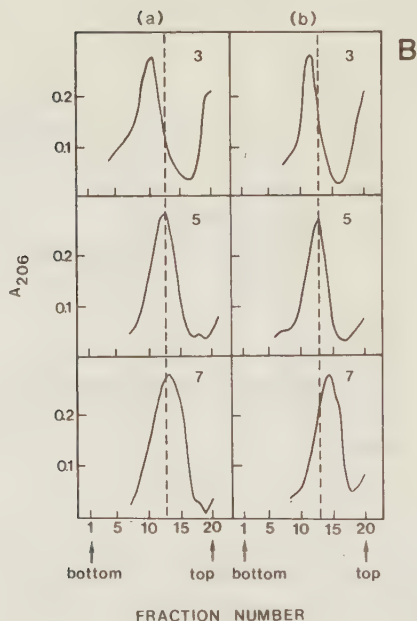
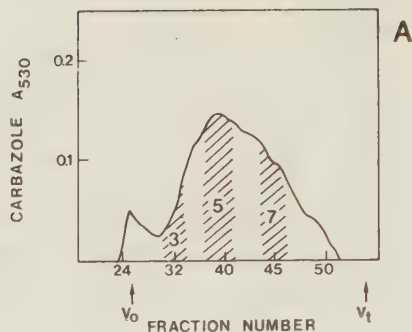


Fig.4A. Fractionation of proteoglycan monomers on a Sepharose Cl-2B column eluted with 4 M-ganidinium chloride. The fractions indicated were pooled, dialysed against sodium acetate and distilled water, freeze-dried and used for zonal rate centrifugation.

Fig.4B. Zonal rate sedimentation of the samples recovered from the Sepharose Cl-2B column shown in fig.4A. Experimental details are given in the text. The samples were centrifuged in 10-30% (a) and 10-50% (b) sucrose gradients.

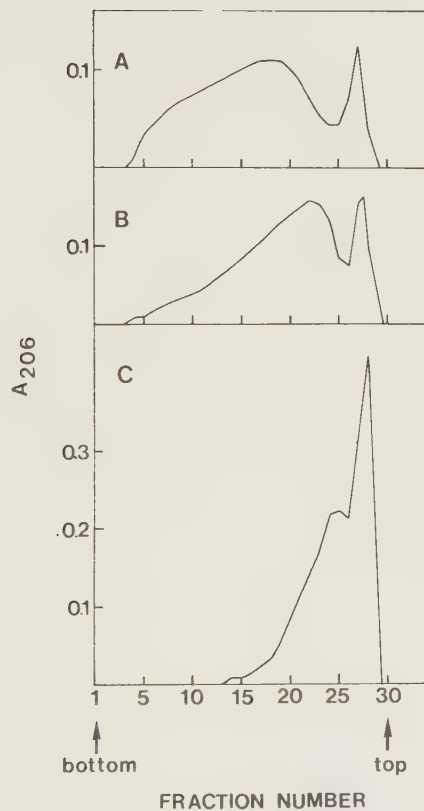


Fig.5. Zonal rate sedimentation of A1-fractions prepared from different cartilages. Experimental details are given in the text. A1-fractions from bovine nasal cartilage (A), bovine tracheal cartilage (B) and bovine articular cartilage (C) were centrifuged for 36 min in 10-50% sucrose gradients.

CARTILAGE PROTEOGLYCAN AGGREGATE FORMATION

Role of Link Protein

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ABSTRACT

Cartilage proteoglycan aggregate formation was studied by zonal rate centrifugation in sucrose gradients. Proteoglycan aggregates, monomers and proteins could be resolved. It was shown that the optimal proportion of hyaluronic acid for proteoglycan aggregates formation was about 1% of proteoglycan dry weight. The reaggregation of dissociated proteoglycan aggregate A1-fraction was markedly concentration dependent and even at 9 mg/ml only about 90% of the aggregates were reformed. The lowest proportion of link protein, required for maximal formation of link stabilized proteoglycan aggregates was 1.5% of proteoglycan dry weight. It was separately shown that link protein cosedimented with the proteoglycan monomer. By competition with isolated hyaluronic acid binding region fragments, a proportion of the link proteins was removed from the proteoglycan monomers, indicating that the link protein binds to the hyaluronic acid binding region of the proteoglycan monomer.

INTRODUCTION

Several cartilage proteoglycan monomers can interact with hyaluronic acid to form aggregates (Hardingham & Muir, 1972). The hyaluronic acid binding region of the proteoglycan, can specifically bind to a decasaccharide or larger sequence of hyaluronate (Heinegård & Hascall, 1974a; Hascall & Heinegård, 1974b; Christner *et al.*, 1978; Hardingham, 1979). Although the K_D of the interaction is in the order of 10^{-8} (Christner *et al.*, 1978; Cleland, 1979; Nieduszynski *et al.*, 1980) an additional protein, the link protein is present to stabilize the interaction. Present, indirect, data indicate that the link protein interacts both with the proteoglycan monomer and with the hyaluronate (Hascall & Heinegård, 1974b; Hardingham, 1979).

The present study was initiated to study some of the parameters important for proteoglycan aggregation. Ratios of link protein and hyaluronic acid to proteoglycan, optimal for aggregate formation were determined. The interaction of link proteins with proteoglycan monomers was studied.

MATERIALS AND METHODS

Proteoglycan monomers (Al-D1-fraction) and aggregates (Al-fraction) were isolated as described elsewhere (Heinegård, 1972; Heinegård & Hascall, 1979). Hyaluronic acid binding region fragment was isolated after trypsin digestion of proteoglycan Al-fraction as described elsewhere (Heinegård & Axelsson, 1977; Heinegård & Hascall, 1974a).

Link proteins were prepared from the top fraction (Al-D4) of the dissociative CsCl density gradient of the Al-fraction. The Al-D4 was recentrifuged in a CsCl - 4 M guanidinium chloride gradient with a starting density of only 1.35 g/ml to remove any remaining proteoglycans. The top fraction was chromatographed on Sephadex G-200, eluted with 4 M guanidinium chloride, essentially as described previously (Heinegård, 1972; Heinegård & Hascall, 1974a), to yield a pure link protein

fraction. High molecular weight hyaluronic acid (Healon^R) was a gift from Pharmacia AB, Uppsala, Sweden. Hyaluronic acid oligosaccharides were prepared from hyaluronic acid by digestion with testicular hyaluronidase followed by gel chromatography on Sephadex G-50 as described elsewhere (Hascall & Heinegård, 1974b).

Zonal rate centrifugation in sucrose gradients was done as described in a companion paper (Franzén *et al.*, 1981). The MSE 6 x 4.2 ml swing out rotor was used at 50,000 rpm (240,000 g; r_{av} 8.802 cm and 20°C) for the times indicated.

Aggregation of proteoglycans with hyaluronic acid

The sedimentation properties of complexes of hyaluronic acid and proteoglycan monomers were studied. A proteoglycan monomer (6 mg/ml) stock solution in 0.15 M sodium chloride, 0.005 M sodium phosphate, pH 7.0 was prepared. Aliquots (50 μ l) were incubated with 50 μ l of the buffer, containing variable amounts of hyaluronic acid for 12 h at 4°C. The solutions were layered on top of 10-50% sucrose, 0.5 M sodium chloride gradients and centrifuged for 36 min.

Reaggregation of proteoglycans

The concentration dependence of proteoglycan reaggregation was determined. Proteoglycan A1-fraction was dissolved at 0.3-9 mg/ml in 4 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8. The samples (200 μ l) were dialysed in a constantly rotating microdialysis apparatus. The apparatus had 10 chambers each having a flat dialysis membrane with a surface area of about 1 cm². Dialysis was first against 1500 ml of 0.5 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8, for 24 h and then against 1500 ml of 0.15 M sodium chloride, 0.005 M sodium phosphate, pH 7.0, for another 24 h. The samples were, when required, adjusted to constant volume and centrifuged in 10-50% sucrose, 0.5 M sodium chloride, 0.005 M sodium phosphate, pH 7.0 gradients for 36 min. The amounts of aggregate and monomer, respectively, were determined as the area of the respective peak in the 206 nm scan.

Dependence of aggregate stability on proportion of link proteins

A stock solution of nasal cartilage proteoglycan monomer (Al-D1) at 6 mg/ml in 4 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8, was prepared. Aliquots (100 μ l), containing 600 μ g, were mixed with equal volumes of 4 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8, containing from 0 to 24 μ g of link proteins. The mixtures, then, contained 3 mg/ml of proteoglycan and link protein at 0-4% (w/w) of the proteoglycan. After dialysis against 1500 ml of 0.5 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8 for 12 h at 4°C, a constant amount of hyaluronic acid (5.4 μ g or 0.9% of proteoglycan dwt) was added to each sample. The samples were dialysed against 1500 ml of 0.15 M sodium chloride, 0.005 M sodium phosphate, pH 7.0, for 24 h at 4°C. One set of 100 μ l aliquots representing each concentration of link protein, was incubated with hyaluronic acid oligosaccharides containing 7 disaccharide units (HA-14) to compete for proteoglycan bound to hyaluronic acid but not stabilized by link proteins (Hascall & Heinegård, 1974b; Björnsson & Heinegård, 1981). Freeze-dried HA-14 (27 μ g) was added to each sample (10 times the amount of hyaluronic acid). They were incubated for 12 h at 4°C prior to centrifugation in 10-50% sucrose, 0.5 M sodium chloride, 0.005 M sodium phosphate, pH 7.0 gradients for 36 min.

Interaction of link protein with proteoglycan monomer

Stock solutions of proteoglycan monomer (fraction Al-D1), 10 mg/ml, link protein, 1 mg/ml, bovine serum albumin, 1 mg/ml and isolated hyaluronic acid binding region, 1 mg/ml were prepared in 4 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8. A series of mixtures were made, all having a constant concentration of proteoglycan monomer (5 mg/ml) and link protein (0.05 mg/ml; 1% of proteoglycan dwt). In addition they contained albumin (0.1 mg/ml; 2% of proteoglycan dwt), or isolated hyaluronic acid binding region (0.1 mg/ml and 0.2 mg/ml respectively; 2% and 4% of proteoglycan dwt). The solutions were dialysed in the microdialysis apparatus against 1500 ml of

0.5 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8, for 24 h at 4°C and then against 1500 ml of 0.15 M sodium chloride, 0.005 M sodium phosphate, pH 7.0, for 24 h at 4°C. The dialysed solutions were diluted with an equal volume of the buffer used for dialysis and aliquots (100 µl); containing 250 µg of proteoglycan, were layered on 10-30% sucrose, 0.5 M sodium chloride, 0.005 M sodium phosphate, pH 7.0, and centrifuged for 4 h at 20°C. The tubes were emptied with continuous monitoring at 206 nm and 200 µl fractions were collected. Aliquots (triplicates) were taken for quantitation of link proteins by enzyme linked immunosorbent assay. The assay (details to be published) includes a preincubation of the sample in 0.8% (w/v) SDS and subsequent formation of mixed micelles of the unbound SDS by addition of ten times excess (w/w) of Triton X-100 (Chua & Blomberg, 1979). The sample is then preincubated with rabbit anti-link anti-body (Wieslander & Heinegård, 1980) and aliquots are transferred to microtiter plate wells, coated with link protein. After incubation for 1 h, the wells are washed and anti-rabbit IgG conjugated with alkaline phosphatase is added. The amount of anti-IgG bound to the well is measured as the enzyme activity and is inversely proportional to the amount of link protein in the sample over a range from about 0.0001 µg/ml to 10 µg/ml.

RESULTS AND DISCUSSION

Hyaluronic acid-proteoglycan monomer interaction

Zonal rate sedimentation centrifugation is very well suited for studies on formation of proteoglycan aggregates, in particular since the relative proportion and size of aggregates formed can be determined simultaneously. In a set of experiments the minimal proportion of hyaluronic acid required to form maximal amount of hyaluronic acid - proteoglycan complex was determined. Proteoglycan monomers were mixed with various amounts of high molecular weight hyaluronic acid, incubated and centrifuged in 10-50% sucrose, Fig. 1. Hyaluronic acid complexes form the broad faster sedimenting peak, while the

proteoglycan monomers remain as a relatively sharp peak near the top of the gradient, Fig. 1. The size of the aggregates remained rather constant up to a proportion of 1% hyaluronic acid (of proteoglycan dwt), while at larger proportions of hyaluronic acid, the size of the aggregates progressively decreased, Fig. 1. The largest proportion of complexes was obtained with 0.9 to 1.05% (w/w) hyaluronic acid. The data indicate that at these concentrations the hyaluronic acid is maximally saturated with proteoglycan monomers and all monomers capable of binding are bound to the hyaluronic acid. When the hyaluronic acid concentration is further increased, the number of proteoglycan monomers bound to each hyaluronic acid progressively decreases and the smaller aggregates formed sediment slower, Fig. 1. The optimal concentration of hyaluronic acid for complex formation with cartilage proteoglycan monomers has previously been found to be about 1% (Hardingham & Muir, 1972; Hascall & Heinegård, 1974a) in accordance with the present data.

Concentration dependence of reassociation of link stabilized proteoglycan aggregates

The concentration dependence of the reaggregation of proteoglycan aggregates (A1-fraction) was determined, Fig. 2. Nasal cartilage A1-fraction was dissociated in 4 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8 and diluted to concentrations ranging from 0.3 mg/ml to 9 mg/ml. The solutions were brought to associative conditions by dialysis as described in the Experimental Section.

As a control nasal cartilage proteoglycan monomers, dissolved in 4 M guanidinium chloride, were mixed with high molecular weight hyaluronic acid (0.9% of proteoglycan dwt). The solutions were diluted to give a set of proteoglycan concentrations from 0.125 mg/ml to 3 mg/ml. The samples were dialysed as described for the A1-fractions. All samples were centrifuged in 10-50% sucrose gradients for 36 min and the proportions of aggregate and monomer were determined as the area under the

respective peak, Fig. 2. Assuming a proteoglycan molecular weight of 2.5×10^6 and a dissociation constant of about 10^{-8} , for the proteoglycan-hyaluronic acid interaction, maximal complex formation should be obtained even at proteoglycan concentrations of 0.125 mg/ml. Expectedly then, mixtures of proteoglycan monomer (0.125-3 mg/ml) and hyaluronic acid contained a constant amount (80-85%) of complex, Fig. 2. Proteoglycan Al-fraction, which in addition contained the link proteins, unexpectedly reaggregated much less efficiently at low concentrations, Fig. 2. At the lowest concentration of 0.3 mg/ml only about 30% of the proteoglycans were aggregated, compared with 83% for the original Al-fraction. At concentrations from 1 mg/ml to about 9 mg/ml increasing amounts of aggregates were obtained. Surprisingly, even at 9 mg/ml only about 75% of the proteoglycans were aggregated. The proteoglycan concentration in the cartilage extract used to prepare proteoglycan Al-fraction is usually no more than 5 mg/ml and yields of aggregate are nevertheless about 85%. Experiments to improve reaggregation of the Al-fraction by changing the dialysis conditions and the geometry of the dialysis bag were unsuccessful. It is possible that the more efficient reaggregation in the original extract is due to the presence of tissue factors promoting aggregation, or more likely do the link proteins renature incompletely on dialysis to associative conditions.

Dependence of aggregate stability on proportion of link protein

Taking into account the concentration dependent reaggregation and using optimal concentration of hyaluronic acid, the amount of link protein required to stabilize aggregates was investigated. The concentration of proteoglycan used was 3 mg/ml. Further increases of the proteoglycan concentration would have only limited effect on the proportion of aggregates formed. Proteoglycan monomer (Al-Dl-fraction) and link protein were dissolved in 4 M guanidinium chloride and mixed to give link protein proportions from 0 to 4% of proteoglycan dry weight. The solutions were dialysed to associative conditions as de-

scribed in the Experimental Section. A constant amount of high molecular weight hyaluronic acid (0.9% of proteoglycan dry weight) was then added to each solution. Consequently only the concentration of link protein was varied. Samples representing each concentration of link protein were incubated with hyaluronic acid oligosaccharides (HA-14, 10 times the amount of hyaluronic acid in the sample). The oligosaccharides will displace proteoglycan monomers from high molecular weight hyaluronic acid unless the interaction is stabilized by link proteins (Heinegård & Hascall, 1974b; Björnsson & Heinegård, 1981). Therefore all aggregates observed upon centrifugation of the samples in the sucrose gradients are link-stabilized. A progressive increase of the size and the amount of stable aggregates, was obtained when the link protein concentration was increased to 1.5%, of proteoglycan monomer dry weight, while further increase did not significantly change the pattern, Fig. 3. The optimal concentration of 1.5% of link protein is close to the proportion estimated using fragmentation of proteoglycan aggregates (Heinegård & Hascall, 1974a).

Binding of link protein to proteoglycan monomer

The interaction between proteoglycan monomer and hyaluronic acid is well characterized (Hardingham & Muir, 1972; Hascall & Heinegård, 1974a; Hascall & Heinegård, 1974b; Christner *et al.*, 1978; Cleland, 1979; Nieduszynski *et al.*, 1980). Evidence indicate that the link proteins can interact with proteoglycan monomers (Baker & Caterson, 1979; Heinegård & Hascall, 1979; Hardingham, 1979; Poole *et al.*, 1980). It is possible that the hyaluronic acid binding region of the proteoglycan monomer is involved in this interaction.

Proteoglycans carrying bound proteins can be separated from other proteins by zonal rate centrifugation (Franzén *et al.*, 1981). This technique can therefore be used to discern whether or not link proteins cosediment with the proteoglycans indicating binding and whether or not any bound link proteins can be removed by competition with proteoglycan substructures,

primarily the hyaluronic acid binding region. A number of samples in 4 M guanidinium chloride containing proteoglycan monomers and link proteins were prepared. The proportion of link proteins, 1% of proteoglycan dwt, is below saturation and was chosen to ensure binding of all link proteins. Hyaluronic acid binding region fragment was added at two concentrations (2% and 4% of proteoglycan dwt) to compete for binding of link proteins. As a control bovine serum albumin (2% of proteoglycan dwt) was added to another sample. All solutions were dialysed to associative conditions and centrifuged in sucrose gradients as described in the Experimental Section. The distribution of protein and proteoglycan in the tube was monitored using the absorbance at 206 nm and the distribution of link protein was determined using ELISA. The profile of the proteoglycan monomer was very similar in all cases regardless of whether or not link protein had been added (not shown). The sample containing only proteoglycan monomer and link proteins showed the same type of profile as the proteoglycan alone with no slow sedimenting protein component, indicating binding of the link proteins (not shown). The sample containing proteoglycan monomer, link protein and albumin in addition showed a slow sedimenting component corresponding to the albumin, Fig. 4a. ELISA for link protein demonstrated that the link proteins sedimented with the proteoglycan monomer, Fig. 4a. The total amount of link protein in the gradient was 2.89 μg as determined by ELISA, in reasonable agreement with the 2.5 μg that was originally added. No link protein was detected in the slow sedimenting protein component. Therefore all the link protein had bound to the proteoglycan monomer. The samples containing proteoglycan monomer, link protein and 2% or 4% of hyaluronic acid binding region fragment, showed similar profiles of proteoglycan monomer. In addition a slow sedimenting component representing the added hyaluronic acid binding region was observed, Fig. 4b and c. The ELISA showed that all link proteins present sedimented with the proteoglycan monomers, Fig. 4b and c. The amounts of link protein, however, were much lower, 1.52 μg (2% added hyaluronic acid binding region fragment) and 1.45 μg (4% added hyaluronic acid

binding region fragment) compared with the expected 2.5 μ g of link protein. Consequently the hyaluronic acid binding region, in contrast to albumin, was capable of competing for binding of link protein. The complex of link protein - hyaluronic acid binding region was not recovered, since no link protein sedimented at the expected position of the hyaluronic acid binding region. Corroborating evidence was the unexpected low recovery of hyaluronic acid binding region fragment particularly when added at lower concentration, Fig. 4b and c. Isolated link protein has a low solubility in low salt solutions (Tang *et al.*, 1979) and is often lost on surfaces when not bound to proteoglycans. The observed bimodality of the link protein distribution, Fig. 4, may represent different binding to the two aggregating supopulations of cartilage proteoglycans, which can be partially separated using zonal rate centrifugation in sucrose gradients (Heinegård *et al.*, 1981).

The data provide strong evidence that the link proteins bind to the hyaluronic acid binding region of the proteoglycan monomer. It should be born in mind, however, that the hyaluronic acid binding region used, had been prepared by fragmentation of the proteoglycan, and may not contain all interacting structures.

GENERAL DISCUSSION

It has been shown that zonal rate centrifugation in sucrose gradients is a useful tool in studies of interactions in proteoglycan aggregation. It appears that proteoglycan monomers interact with hyaluronate at an optimal ratio of about 100:1, respectively, corroborating previous data (Hardingham & Muir, 1972; Hascall & Heinegård, 1974a). The interaction is stabilized by link proteins at an optimal concentration of 1.5% of proteoglycan dry weight, which corresponds to one link protein per proteoglycan monomer, assuming molecules weights of 45,000 and $2.5 \cdot 10^6$, respectively. The link protein was shown to interact with the isolated hyaluronic acid binding region of

the proteoglycan monomer. Since it also interacts with isolated hyaluronic acid (unpublished) the binding of proteoglycans to hyaluronate in the presence of link proteins is trifunctional.

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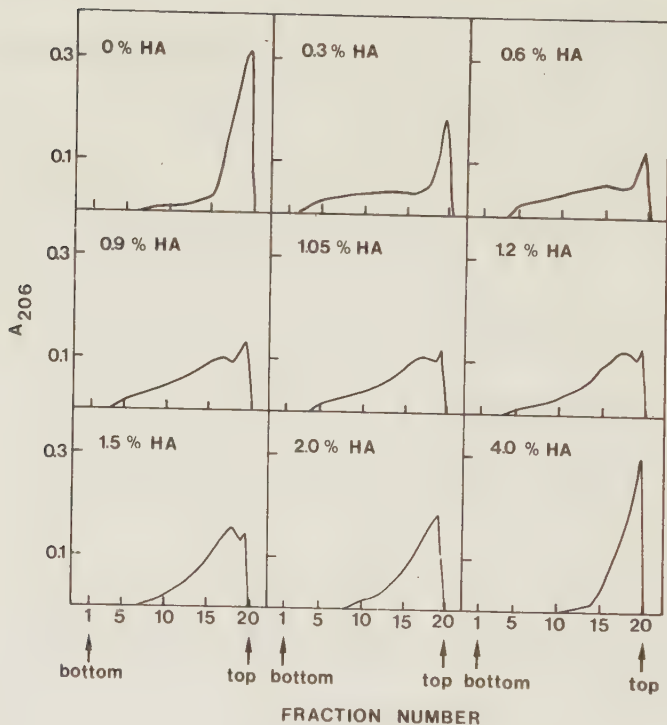


Fig.1. Zonal rate sedimentation for 36 min in 10-50% sucrose gradients of a constant amount (300 ug) of proteoglycan monomer with various concentrations of high molecular weight hyaluronic acid (up to 4% of proteoglycan weight). Experimental details are given in the text..

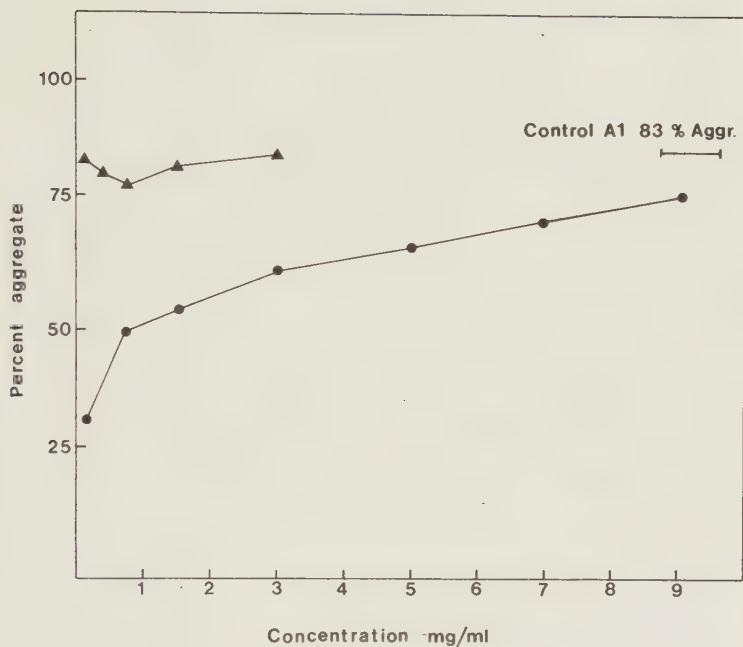


Fig.2. Zonal rate sedimentation for 36 min in 10-50% sucrose gradients of nasal cartilage proteoglycan aggregates (A1-fraction) dissolved in 4 M guanidinium chloride at concentrations of 0.125 mg/ml to 9 mg/ml.

The proteoglycans were reaggregated as described in Experimental Section.

The proportion of proteoglycan aggregates (●) were calculated from the area of the peaks for aggregate and monomer, respectively. As control

the proportion of aggregate in the original A1-fraction is shown (—).

For comparison the proportion of aggregate formed when proteoglycan monomer

at 0.125 mg/ml to 3 mg/ml were mixed with a constant proportion of high

molecular weight hyaluronic acid (0.9% of proteoglycan weight) is shown (▲).

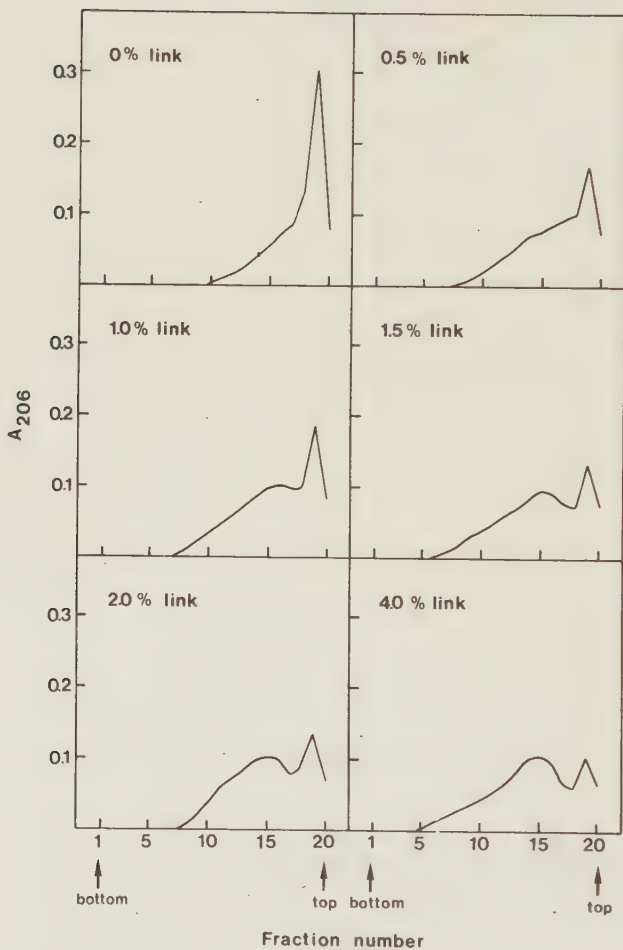


Fig.3. Zonal rate sedimentation in 10-50% sucrose gradients for 36 min of aggregates formed by incubating a constant proportion of proteoglycan monomer and hyaluronic acid with a variable amount of link protein (from 0 to 4% of proteoglycan dry weight). Experimental details are given in the text. Prior to centrifugation samples were incubated with an excess of hyaluronic acid oligosaccharides (HA-14) to dissociate any hyaluronic acid-proteoglycan monomer aggregates not stabilized by link proteins.

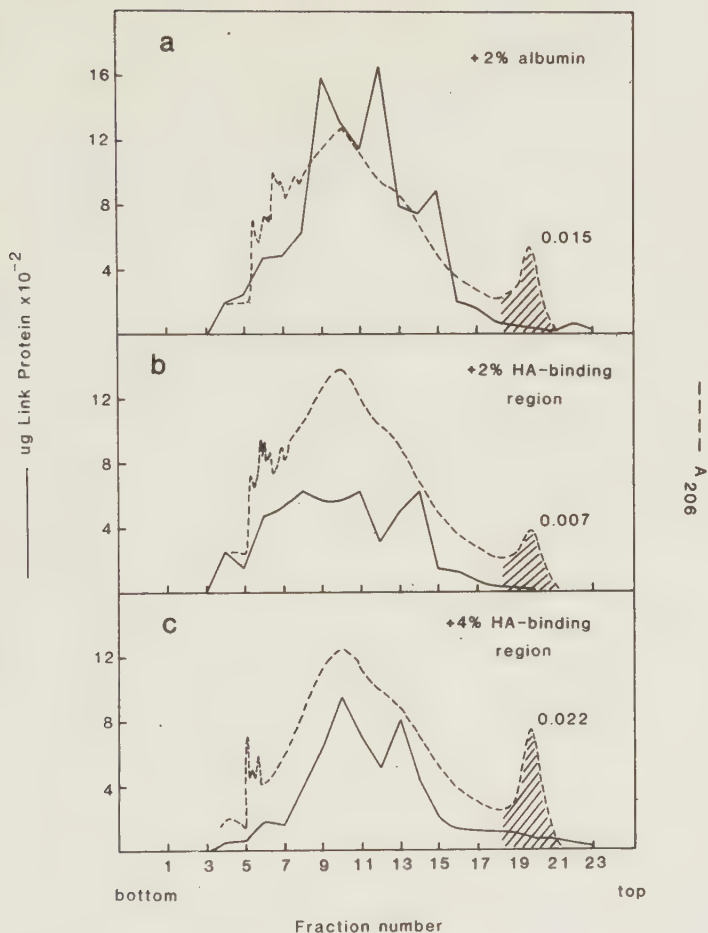


Fig.4. Zonal rate centrifugation in 10-30% sucrose gradients of mixtures of (a) proteoglycan monomer (250 ug), link protein (2.5 ug) and bovine serum albumin (5 ug), (b) proteoglycan monomer (250 ug), link protein (2.5 ug) and isolated hyaluronic acid binding region (5 ug) and (c) proteoglycan monomer (250 ug), link protein (2.5 ug) and isolated hyaluronic acid binding region (10 ug). Experimental details are given in the text. The gradients were monitored for absorbance at 206 nm (---) and for content of link proteins using ELISA (—). The area of the indicated peak is a measure of the amount of the protein not bound to proteoglycan.

v

ASSEMBLY OF PROTEOGLYCAN AGGREGATES IN CULTURES OF
CHONDROCYTES FROM BOVINE TRACHEAL CARTILAGE

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SYNOPSIS

The assembly of proteoglycan aggregates in chondrocyte cell cultures was examined in pulse-chase experiments with the use of [35 S]sulphate for labelling. Zonal rate centrifugation in linear sucrose gradients (10-50%, w/v) was used to separate the aggregated proteoglycans from monomers and to assess the size of the newly formed aggregates. The proportion of aggregates stabilized by link protein was assessed by competition with added exogenous aggregate components. The capacity of the proteoglycans synthesized in culture to compete with exogenous nasal cartilage proteoglycans for binding was studied in dissociation-reassociation experiments. The results were as follows. (a) The proteoglycan monomers and the hyaluronic acid are exported separately and combined extracellularly. (b) The size of the aggregates increases gradually with time as the proportion of monomers bound to hyaluronic acid increases. (c) All of the aggregates present at a particular time appear to be link-stabilized and therefore not dissociated by added excess of nasal proteoglycan monomer or hyaluronic acid oligomers. (d) The free monomer is apparently present as complex with link protein. The monomer-link complexes are then aggregated to the hyaluronic acid. (e) The aggregates synthesized *in vitro* and the nasal cartilage aggregates differ when tested for link-stabilization by incubation at low pH. The aggregates synthesized *in vitro* were completely dissociated whereas the cartilage proteoglycans remained aggregated. The results obtained from dissociation-reassociation experiments performed at low pH indicate that the proteoglycan monomer synthesized *in vitro* does not bind the hyaluronic acid or the link protein as strongly as does the nasal cartilage monomer.

INTRODUCTION

The biological function of cartilage as a shock absorber is dependent on the molecular organization of the major components of the tissue, proteoglycans and collagen. The proteoglycan aggregates occupy large domains inside the collagen network and immobilize the water in the tissue. Proteoglycan aggregates may contain 50-100 proteoglycan monomers non-covalently bound to hyaluronic acid (for references see Hascall & Heinegård, 1979). Each aggregate, when extended in solution, has a hydrodynamic volume approaching the volume of the chondrocyte. Two central problems pertaining to the biology of cartilage are therefore how the chondrocyte handles these large molecules and how proteoglycans move through the matrix to be deposited at a required site. A primary question is whether the aggregates are assembled in the cell or in the matrix.

In the aggregate the proteoglycan monomers bind to hyaluronic acid by the hyaluronic acid-binding region of the protein core of the monomer. The interaction is stabilized by link protein. The aggregates may thus be link-stable or link-free. The proteoglycan monomer in the link-free aggregate is open to exchange with free monomer or hyaluronic acid, whereas the monomers in link-stable aggregates appear irreversibly bound to the hyaluronic acid. Thus, when either component of the aggregate is added in large excess, the monomers in link-free aggregates are displaced when the new equilibrium takes place (Kimura *et al.*, 1979). Kimura *et al.* (1979), using rat chondrosarcoma cells in monolayer cultures, have shown that link-stable aggregates are formed extracellularly, probably via a monomer link-protein precursor (Kimura *et al.*, 1980).

In a previous paper (Björnsson & Heinegård, 1981a) we described the procedures used to isolate and culture foetal bovine chondrocytes. The cells, even in the absence of serum, synthesized a cartilage proteoglycan with all typical features (Björnsson & Heinegård, 1981b). The proteoglycans were present in the medium as aggregates of similar size as those from the

tracheal tissue. Both link proteins present in aggregates from the tissue were identified in the aggregates from the cell cultures.

The aim of the present investigation was to monitor the assembly of such proteoglycan aggregates in cultures of foetal bovine chondrocytes in the absence of exogenous proteins, which might affect the aggregation. Zonal rate centrifugation in sucrose gradients was used to separate aggregates and monomers and to determine the size of the aggregates.

EXPERIMENTAL

Materials. Collagenase CLS II was obtained from Worthington Biochemical Corp. (Freeholdt, NJ, U.S.A.). Ham's F12 medium was from GIBCO (Grand Island, NY, U.S.A.). Benzoylpenicillin was from Astra (Stockholm, Sweden). Streptomycin sulphate was from Merck (Darmstadt, Germany). Polypropylene vials (1.5 ml, no. 3810, and 2.2 ml, no. 3812) were obtained from Eppendorf, (Hamburg, Germany). Carrier-free [35 S]sulphate was from The Radiochemical Centre (Amersham, Bucks., U.K.). Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Hyaluronic acid (Healon) was a gift from Pharmacia Fine Chemicals (Uppsala, Sweden). Dextran sulphate and Sepharose 2B were purchased from Pharmacia. Instagel was from Packard Instrument Co. (La Grange, IL, U.S.A.).

Proteoglycan aggregates (NC-A1 fraction) and monomers (NC-A1-D1 fraction) were prepared from bovine nasal cartilage as described previously (Heinegård, 1972; Heinegård & Hascall, 1979).

Hyaluronic acid oligomers were isolated from partial digests of hyaluronic acid with testicular hyaluronidase by gel chromatography on Sepharose G-50 as described previously (Hascall & Heinegård, 1974). HA₁₄ fraction denotes an oligomer of hyaluronic acid containing 14 monosaccharides.

Preparation of cell cultures. Chondrocytes were isolated from foetal bovine tracheas as described previously (Björnsson & Heinegård, 1981a). After incubation for 1-2 days at 37°C, the cells were collected by centrifugation and suspended in Ham's F12 medium (without streptomycin sulphate). The cell suspension was pipetted into Eppendorf vials and incubated at 10×10^6 cells/ml on an Adams nutator with orbital motion for 1 h before being labelled with [^{35}S]sulphate.

Pulse experimental protocol. The cells (20×10^6 in 2 ml) were pulsed with [^{35}S]sulphate (5 μCi in 50 μl) for times between 2.5 and 60 min. The pulse was terminated by pelleting the cells for 30 s in an Eppendorf centrifuge (model 5412) operated with low voltage (90 V).

The medium was rapidly withdrawn with a syringe, and the cell pellet was resuspended in 1 ml of fresh non-radioactive medium and again pelleted as above. All the cell pellets were frozen in liquid N_2 and kept at -60°C until extracted.

The cells were extracted with 1 ml of 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8, containing 10 mg of NC-A1 fraction/ml for 14 h at 4°C. After addition of 5 ml of the same solution to the extract, any particulate material was pelleted by centrifugation at 100 000 g_{av} for 1 h. The supernatants were dialysed against 10 vol. of 50 mM-sodium acetate buffer, pH 5.8, containing proteinase inhibitors (0.1 M-6-aminohexanoic acid, 10 mM- Na_2EDTA and 5 mM-benzamidine hydrochloride). Associative density-gradient centrifugation performed as described previously (Heinegård, 1972; Heinegård & Hascall, 1979) was used to isolate the proteoglycans from the reassociated cell extract. The A1 fractions were chromatographed on a Sepharose 2B column (150 cm x 0.9 cm) eluted with 0.5 M-sodium acetate buffer, pH 7.0. Fractions (1.50 ml) collected were analysed for radioactivity and uronic acid contents (Heinegård, 1973).

Pulse-chase protocol. The cells (6×10^6 in 0.6 ml) were pulsed with [^{35}S]sulphate (12 μCi in 25 μl) for 15 min. The pulse was terminated by the addition of 600 μl of fresh unlabelled medium containing 20 mM- Na_2SO_4 . The cells were pelleted as described above and the medium was discarded. The pellets were suspended in 1200 μl of fresh medium containing 10 mM- Na_2SO_4 and again pelleted. Finally, the cells were resuspended in 600 μl of the same medium (Scheme 1). The time between the end of the pulse and the start of the chase was approx. 1.5 min. By this procedure the amount of unincorporated [^{35}S]sulphate remaining in the medium was decreased to below 0.02% of the concentration during the pulse. The cells were incubated as above, and the chase was continued for the times stated in the particular experiment. At the end of the chase the cells were pelleted and the medium was withdrawn. The distribution of [^{35}S]sulphate was studied in sucrose gradients, either immediately at the end of the chase or after incubation with added exogenous monomer on hyaluronic acid or hyaluronic oligosaccharide. In some experiments dissociation-reassociation of the proteoglycan aggregates was performed as described below.

Two pulse-chase experiments are discussed in the text. The first experiment was performed with either 15 min or 24 h of chase after the 15 min pulse. At the end of the 15 min chase, one sample (200 μl) was incubated at 37°C for 24 h in the absence of cells. Another sample (200 μl) was immediately layered on top of a sucrose gradient prepared in advance and centrifuged at 240 000 g_{av} for 3 h. The two samples incubated with and without cells for 24 h were centrifuged as above.

The second experiment was performed with 15, 30, 45, 90, 180 or 360 min of chase after the 15 min pulse, and analysed as described below.

Size of aggregates. Portions (50 μl) of the samples were layered on sucrose gradients and centrifuged at 240 000 g_{av} for 36 min immediately after the chase. After sedimentation for only 36 min, the proteoglycan monomer is only partly

resolved from the unincorporated label at the top of the gradient. Therefore the amount of radioactivity incorporated into proteoglycans was obtained from an experiment where samples of the same medium were dialysed so that the unincorporated label was removed before centrifugation in the sucrose gradient. The proportion of monomers that had aggregated was determined as the radioactivity sedimenting as aggregates (fractions 1-23 in Fig. 3) divided by the total proteoglycan radioactivity, determined as the radioactivity sedimenting as monomers (fractions 15-25 in Fig. 5e) after dissociation-reassociation of the sample with excess of nasal proteoglycan monomer, performed as described below.

Stability of the aggregates. Portions of the same samples were incubated for 24 h at 4°C as follows: (1) with added NC-Al-D1 fraction at 3 mg/ml, (2) with HA₁₄ fraction at 0.2 mg/ml and (3) with hyaluronic acid at 0.2 mg/ml; they were then centrifuged at 240 000 g_{av} for 36 min. The proportion of aggregated proteoglycans was calculated as described above.

Dissociation-reassociation. To other portions of the same samples was added an equal volume of 8 M-guanidinium chloride to dissociate the proteoglycan aggregates. Subsequent dialysis to associative conditions was performed in a constantly moving chamber (Franzén *et al.*, 1981b), which allows dialysis of small volumes without changes of volume. Reassociation was performed as follows: (1) without added exogenous proteoglycans, (2) with added NC-Al fraction at 3 mg/ml and (3) with added NC-Al-D1 fraction at 3 mg/ml. Dialysis was performed overnight against a solution containing the proteinase inhibitors discussed above.

pH-stability of the aggregates. Samples were adjusted to pH 4.0 by incubating the medium with 1 vol. of 0.1 M-citric acid, pH 2.5, for 24 h before sucrose-density-gradient centrifugation at pH 4.0.

Analytical methods. The details of preparing and analysing the sucrose gradients, including sedimentation profiles of various proteoglycan preparations, are presented elsewhere (Franzén *et al.*, 1981a). Linear sucrose gradients (3.6 ml) with 10-50% (w/v) sucrose in 0.5 M-NaCl/5 mM-sodium phosphate buffer, pH 7.0 (or 0.5 M-NaCl/1 mM-citric acid buffer, pH 4.0) were prepared in 4.2 ml MSE polycarbonate tubes. The samples (50-200 μ l) were carefully layered on top of the gradient. The gradients were centrifuged in a swing-out MSE 6 x 4.2 ml rotor at 240 000 g_{av} . By 36 min of centrifugation, the aggregates sedimented approximately halfway down the gradient, while the monomers essentially remained at the top of the gradient. Alternatively, the gradients were centrifuged for 3 h, in which case the monomers were recovered at a position halfway down the gradient while aggregates had sedimented to the bottom.

The gradients were frozen and sliced in order to achieve a good recovery of the material sedimenting to the bottom of the tube. The gradients were frozen in an aluminium block, and 200 μ l of 10% (w/v) sucrose containing 0.01% Bromophenol Blue was frozen on top of the frozen gradients. They were mounted in a vice and chilled with solid CO₂, and the bottom 2 mm of the tube was cut off with a saw. A Repette (Jencon) stepwise-dispensing syringe was fixed (with the barrel removed) to the vice with the Teflon piston into the centrifuge tube, facing the frozen Bromophenol Blue solution. The frozen gradient was then stepwise expelled out of the tube, and 2 mm slices were cut with a scalpel. The top layer of 0.01% Bromophenol Blue in 10% sucrose was used to separate the top of the gradient from the piston to assure reproducible slicing of the top. The Bromophenol Blue colour was included to detect any thawing of the gradient during slicing. The slices were analysed for radioactivity. In some experiments the gradients were emptied from the bottom by a glass capillary connected to a peristaltic pump via an LKB Uvicord spectrophotometer to measure the absorbance at 206 nm, as described elsewhere (Franzén *et al.*, 1981a). When required, 0.12 ml fractions were collected and

analysed for radioactivity. Radioactivity was measured in a Packard 2650 scintillation counter, with Instagel as the scintillation medium. The sample volume was increased to 2 ml by addition of water to prevent precipitation of the sucrose on addition of 3 ml of Instagel.

RESULTS AND DISCUSSION

Incorporation of [^{35}S]sulphate

In a previous study, serum-free cultures of foetal bovine chondrocytes were used to monitor for the incorporation of [^{35}S]sulphate into glycosaminoglycans over 6 days (Björnsson & Heinegård, 1981a). Most of the ^{35}S -labelled glycosaminoglycans were recovered soluble in the medium, and only a minor portion (5% of total) was associated with the cells. The times selected were too sparse to allow any conclusions regarding the accumulation of the cellular pool. In order to study whether one population with time transforms into another, pulse-chase experiments are usually applied. An experiment with continuous pulse was performed to determine the minimal length of the pulse needed to equilibrate the cells with [^{35}S]sulphate and to obtain a constant rate of export of the proteoglycans. The cells were separated from the medium at the times indicated in Fig. 1 and extracted with 4 M-guanidinium chloride. Proteoglycans were then prepared from the medium and the extract by associative gradient centrifugation as described in the Experimental section. The cellular pool was equilibrated with [^{35}S]sulphate within 10 min of pulse, though it again increased slowly at later times (Fig. 1). This second phase probably reflects the accumulation of the pericellular pool at the surface of the cells. The medium pool increased linearly from 10 min, coinciding with the equilibration of the cellular pool. Pulse-chase experiments were therefore performed with a pulse of 15 min in order to saturate the cellular pool with [^{35}S]sulphate.

Aggregatability of monomers

The 4 M-guanidinium chloride extract of the cells from the continuous-pulse experiment discussed above was reassociated in the presence of nasal cartilage proteoglycan aggregates as described in the Experimental section. The bottom fractions from the associative CsCl-density-gradients were chromatographed on Sepharose 2B (not shown). Already at 15 min of a [^{35}S]sulphate pulse approx. 75% of the radioactive proteoglycan monomers were eluted in the void volume. The 4 M-guanidinium chloride extract of the cells, that constitutes 95% of the cellular [^{35}S]sulphate labelled material, contains proteoglycan monomers derived from the intracellular pool. On reassociation in the presence of nasal cartilage proteoglycan aggregates the radioactive cell proteoglycan monomers and the non-radioactive nasal cartilage proteoglycan monomers will form mixed aggregates. Thus the intracellular pool consists of molecules that already have the capability of interacting with hyaluronic acid, even though binding might not occur until a later stage.

Localization of the site of aggregation

In order to determine the site of aggregation, whether extra- or intra-cellular, a pulse-chase experiment with [^{35}S]sulphate was performed with either 15 min or 24 h of chase. At the end of the chase the proportion of aggregates and monomers was determined by zonal rate centrifugation in sucrose gradients as described above. The gradients were centrifuged at $240\,000\,g_{av}$ for 3 h. After 15 min of chase (Fig. 2a), only 2.6% of the incorporated radioactivity was recovered at the bottom of the tube sedimenting as aggregates, and the rest was in the middle of the tube at the position of monomers. The unincorporated label remained at the top of the gradient and is not included in the calculations. After 24 h of chase the proportion [^{35}S]sulphate in aggregates was 79% (Fig. 2b). Complexes of hyaluronic acid and proteoglycan monomers not stabilized by link proteins are also stable in sucrose gradients and they sedimented as aggregates (Franzén *et al.*, 1981b). Therefore,

the absence of any radioactive component sedimenting faster than the monomer at the short chase time suggests that the proteoglycan monomers and the hyaluronic acid are exported separately and are combined extracellularly.

In a similar experiment the medium after 15 min of chase, was incubated at 37°C in the absence of cells. After 24 h of incubation the amount of radioactivity sedimenting as aggregates was 65%, showing that the presence of cells is not required for the assembly of the exported monomers into aggregates. The amount of radioactivity sedimenting as proteoglycan (aggregates plus monomers) was nearly identical at 15 min of chase (12 848 cpm) and 24 h of chase (14 256 cpm). Thus 90% of the incorporated radioactivity recovered in the medium at 24 h of chase is already secreted within 15 min. The amount of free [³⁵S]sulphate left over from the pulse and recovered at the top of the sucrose gradients constitutes about 45% (44.3 and 43.9% respectively) of the total radioactivity present in the medium, when the pulse-chase protocol described in the Experimental section is used.

Size of aggregates

The extracellular assembly of aggregates might proceed along two different lines. Each aggregate may be assembled separately and completed to achieve its final size before the next aggregate is formed. Alternatively, the different components of the aggregates, namely monomers, hyaluronic acid molecules and link proteins, are combined at random, forming aggregates that gradually increase in size as the proportion of monomers bound to hyaluronic acid increases.

A pulse-chase experiment with a 15 min pulse of [³⁵S]sulphate followed by 15, 30, 45, 90, 180 and 360 min of chase was undertaken to discriminate between these two possibilities. The pulses were initiated at different times, so that the end of all chase times occurred simultaneously. Samples of each medium were immediately layered on sucrose gradients prepared in advance. The sedimentation patterns of the aggregates were

compared after 36 min at 240 000 g_{av} (Fig. 3). By 15 min of chase most of the label was recovered at the top of the gradient at the position of monomers. A minor portion of the radioactivity, however, sedimented faster than the monomer peak, which suggests that some monomers (9%) had formed aggregates.

The proportion of the radioactivity sedimenting as aggregates increased with time (Figs. 3 and 7a). The rate of aggregation is highest at the beginning of the experiment (Fig. 7a). One explanation is that the monomers first bound to the hyaluronic acid exert a steric hindrance to the binding of other monomers to the same aggregate. The size of the aggregates also increased with time and can be measured as the proportion of radioactivity recovered in fractions 10-14 compared with the total radioactivity in fractions 2-23 of the sucrose gradient. The size of the aggregates increased linearly for up to 3 h of chase (Fig. 7a) and was then approximately the same as for aggregates isolated from cultures incubated for 6 days (results not shown). With time more of the largest-size aggregates accumulated at the bottom of the tube (Fig. 7a). In a control experiment excess of monomer (3 mg/ml) was added to the cultures at the beginning of the chase. By 6 h of chase all radioactivity was still recovered at the top of the gradient (results not shown). The absence of any aggregates in the presence of excess of monomer in the medium suggests that the exogenous monomer takes part in the equilibrium with the endogenous hyaluronic acid and competes with the endogenous monomer for binding sites. The results indicate that the aggregation occurs at random in the medium, rather than as an ordered assembly of monomers to aggregates.

Stability of the newly formed aggregates

Aggregates link-stable as well as link-free remain stable during sedimentation in sucrose gradients as discussed above. To discriminate between the two forms of aggregates, the following competition experiments were performed. A large

excess of exogenous components was added to the medium in order to displace the monomers in link-free aggregates from the hyaluronic acid. Samples of the medium from the cultures that were pulsed with [35 S]sulphate for 15 min and then chased for 15, 30, 45, 90, 180 or 360 min were incubated for 24 h at 4°C with nasal proteoglycan monomers (3 mg/ml), hyaluronic oligosaccharide (HA 14 fraction, 0.2 mg/ml) or high-molecular-weight hyaluronic acid (0.2 mg/ml). Proteoglycan aggregates and monomers in the incubation mixtures were subsequently separated in sucrose gradients centrifuged at 240 000 g_{av} for 36 min.

Incubation with exogenous monomer. A major proportion of proteoglycan monomers will bind to added hyaluronic acid to form aggregates. Since the bound monomers are in equilibrium with free monomers, an exchange between free and bound molecules occurs continuously. Thus, if a large excess of exogenous monomers is added, most of the aggregated monomers will with time be derived from the exogenous monomers. Proteoglycan monomers in link-stable aggregates will not take part in the equilibrium with free monomer (Kimura *et al.*, 1979). Consequently the proportion of link-stable aggregates can be measured as aggregates remaining after incubation with a large excess of proteoglycan monomers. To assess the proportion of link-stable aggregates samples of the medium from the pulse-chase experiment, discussed above, were incubated with nasal proteoglycan monomers for 24 h and analysed by centrifugation in sucrose gradients (Figs. 4a and 4b; only the 15 min chase and 6 h chase are shown in the Figure). At no time did the exogenous monomer displace the endogenous monomer from the aggregates. Apparently, the endogenous monomers are bound in link-stable aggregates and do not take part in the equilibrium with free monomer. Furthermore, the exogenous monomer does not appear to compete with the endogenous monomer on equal terms, since the proportion of endogenous monomer bound to aggregates actually increased at short chase times (Fig. 7b).

At all times the size of the aggregates was so large that they sedimented to the bottom of the tube at 36 min. The increased size of the aggregates might be explained as binding of exogenous monomers to binding sites on the hyaluronic acid not occupied by endogenous monomers.

Incubation with oligosaccharide (HA₁₄ fraction). An HA₁₄ oligosaccharide can bind one proteoglycan monomer but is not sufficiently large to bind one monomer and one link protein (Hascall & Heinegård, 1974; Kimura *et al.*, 1979). If an excess of HA₁₄ fraction is added to a mixture of proteoglycan monomers and hyaluronic acid, the oligosaccharide will take part in the equilibrium. With time essentially all proteoglycan monomers will be bound to the HA₁₄ fraction. Link-stable proteoglycan aggregates will not take part in the equilibrium with the oligosaccharide.

Samples of the medium from the pulse-chase experiments were incubated with HA₁₄ fraction for 24 h and analysed by sucrose-gradient centrifugation (Figs. 4c and 4d). The proportion of aggregates increased at shorter chase times (Fig. 7b). This is the expected result if each monomer that becomes bound to the endogenous hyaluronic acid is immediately stabilized with link protein, whereas the monomer bound to the oligosaccharide still is in equilibrium with the population of free monomers. Consequently, an ever-increasing proportion of monomers will become part of link-stable aggregates, even in the presence of oligosaccharide (Kimura *et al.*, 1979). It is more obvious by short chase times where the population of not yet aggregated monomers is larger. At later chase times the amount of proteoglycan aggregates remains constant, indicating that nearly all the aggregating monomers are already part of link-stable aggregates at the end of the chase. The aggregates sediment a shorter distance after incubation with oligosaccharide, indicating a smaller size compared with the analysis performed directly at the end of the chase.

Incubation with high-molecular-weight hyaluronic acid. Further tests for presence of link-stable aggregates were done with excess of added high-molecular-weight hyaluronic acid. The exogenous hyaluronic acid will compete with endogenous hyaluronic acid for binding of free monomers. With time all monomers in link-free aggregate will become aggregated to the exogenous hyaluronic acid, with only a few monomers per molecule of hyaluronic acid. Since link-stable aggregates might be formed with exogenous high-molecular-weight hyaluronic acid but not with the oligosaccharide, the presence of hyaluronic acid in excess will inhibit further formation of large aggregates. In contrast, added oligosaccharides are not capable of binding monomer plus link proteins and will therefore only retard the formation of link-stable aggregates with the endogenous hyaluronic acid.

Samples of the medium from the pulse-chase experiment were incubated with hyaluronic acid for 24 h and analysed by centrifugation in sucrose gradients (Figs. 5e and 5f). Surprisingly, the exogenous hyaluronic acid not only competed for binding of free monomers with the endogenous hyaluronic acid, but also effectively displaces the already bound monomers at all chase times (Fig. 7b). Possibly, at the very low concentration of proteoglycans in the chase medium there is a significant exchange between free and bound monomers even in the presence of link proteins. Accordingly, almost all proteoglycan monomers will become bound to the exogenous hyaluronic acid with time. In control experiments it has been shown, that added high molecular weight hyaluronic acid does not affect the sedimentation of proteoglycan aggregates.

Dissociation and reassociation

Proteoglycans from cartilage are usually prepared by extraction of the tissue with 4 M-guanidinium chloride, which dissociates the aggregates and allows the components to diffuse out of the tissue. During the subsequent dialysis to associative conditions the proteoglycan monomers, the hyaluronic acid and the link proteins reassociate. The

procedure demonstrates the reversible denaturation of the proteins involved (Sajdera & Hascall, 1969). The proportion of monomers that reaggregate increases with increasing concentration (Franzén *et al.*, 1981b). Experiments were performed to study the capacity of the newly synthesized monomers to denature reversibly and reassociate.

Reassociation without exogenous proteoglycan. One volume of 8 M-guanidinium chloride was added to samples of the medium from the pulse-chase experiment to dissociate the aggregates. After dialysis to associative conditions, monomers and aggregates were separated by centrifugation in sucrose gradients (Figs. 5a and 5b). The reassociations performed with only the low concentrations of proteoglycans present in the chase medium appear to proceed to near completion (Fig. 7c), although the actual values for aggregates content are somewhat scattered (Fig. 7c). The proportion and size of aggregates, however, were somewhat larger at long chase times compared with short times. This is not unexpected considering that unlabelled proteoglycan synthesized during the chase takes part in the equilibrium.

Reassociation with exogenous proteoglycan aggregates. Proteoglycan A1 fraction (NC-A1 fraction) dissolved in 1 vol. of 8 M-guanidinium chloride was added to samples of the medium from the pulse-chase experiment. After dialysis, monomers and aggregates were separated by centrifugation in sucrose gradients at $240\,000\,g_{av}$ for 1 h (Fig. 5c and 5d). Both the proportion and size of the reassociated aggregates were independent of the length of the preceding chase. Since the mixed aggregates formed were considerably smaller than the native aggregates formed in culture, the proteoglycan aggregates required 1 h of sedimentation for separation from monomers. In addition, the largest-size aggregates sedimenting to the bottom of the tube on direct analysis were not present. The proportion of aggregate was constant, indicating that in the presence of exogenous aggregates the reassociation

proceeds reproducibly. Since the endogenous proteoglycans have equal capacity to form mixed aggregates regardless of time spent in culture, extracellular processing resulting in improved aggregatability is unlikely. The proportion of proteoglycan monomers that reaggregate is concentration-dependent, and at the 3 mg/ml used in this experiment only half of the exogenous proteoglycan is reaggregated (Franzén *et al.*, 1981b; compare also with Fig. 6c below). Proteoglycan A1 fraction contains a proportion of aggregating proteoglycan monomers not bound into aggregates (Heinegård & Hascall, 1979). The proportion of the endogenous monomer recovered in the reassociated mixed aggregate fraction (Fig. 7c) is lower than that in the native aggregates (Fig. 7a). Whether the endogenous monomer is less efficiently renatured during reassociation or the excess of exogenous proteoglycan interferes with the binding of link to the endogenous monomer is not known. Since nearly all link proteins present are of exogenous origin, it is likely that the endogenous monomer can interact with the exogenous link protein. Otherwise the excess of free exogenous monomer would displace all endogenous not link-stabilized monomers. However, the proportion of endogenous monomers aggregated is significantly lower when reaggregated with exogenous aggregates than in the medium after 6 h of chase.

Reassociation with exogenous monomer. Proteoglycan monomers (A1-D1 fraction) were dissolved in 8 M-guanidinium chloride, and 1 vol. of the solution was added to samples of the medium from the pulse-chase experiment. After dialysis, the sedimentation in sucrose gradients was performed at 240 000 g_{av} for 3 h. When reassociation is performed in the presence of a large excess of proteoglycan monomer, the exogenous monomer will compete for the binding sites on the hyaluronic acid and only a negligible portion of the endogenous monomer will become bound. Expectedly, at all chase times the radioactivity sedimented to the position of free proteoglycan monomers (Figs. 5e and 5f). If the endogenous link protein was able to bind only to the endogenous monomer, however, these should

with time became irreversibly bound to the hyaluronic acid, even in the presence of excess of exogenous monomer. Therefore the absence of any radioactive aggregates (Figure 7c) indicates that the endogenous link protein is capable of binding also to the exogenous nasal cartilage proteoglycan monomers. The sedimentation of endogenous monomers shows no variation with time spent in culture, indicating that degradation does not occur during the chase period.

pH stability of proteoglycan aggregates. Previous work (Tang *et al.*, 1979) has shown that link-stable and link-free proteoglycan aggregates differ with regard to stability at low pH. Thus link-free aggregates are dissociated at pH 4.0, whereas link-stabilized aggregates are stable. These results were verified by using sucrose-gradient centrifugation to separate proteoglycan monomers and aggregates. A mixture of proteoglycan monomer (A1-D1 fraction) and hyaluronic acid was incubated at pH 4 for 24 h, and the proportion of aggregates was determined by sucrose-gradient centrifugation (Fig. 6a). Very little absorbance was detected at the position of aggregates. In contrast, proteoglycan A1 fraction incubated at pH 4.0 showed the same sedimentation profile as was obtained at pH 7.0 (results not shown). Samples of the medium from the pulse-chase experiment were adjusted to pH 4.0 and analysed by sucrose-gradient centrifugation. No aggregates were detected at any time (Fig. 6b). It is likely, then, that the aggregates assembled in culture are different from those isolated from the tissue. The link protein may either be synthesized in a form that is less stable at low pH, or the hyaluronic acid-binding region of the monomer may not be able to bind the hyaluronic acid or the link protein in the same way as the tissue-derived monomer. In order to test these two possibilities, proteoglycan A1 fraction in 8 M-guanidinium chloride was added to equal portions of the medium from the pulse-chase experiment. The dissociated proteoglycans were reaggregated by dialysis to associative conditions. After dialysis, the pH was adjusted to 4.0 and the solutions were

incubated for 24 h. Aggregates and monomers were separated in sucrose gradients. The position of the exogenous proteoglycan was monitored as absorbance at 206 nm (Franzén *et al.*, 1981a) and the position of the endogenous proteoglycan as radioactivity (Fig. 6c). The absorbance tracing is typical of a nasal cartilage aggregate preparation, whereas the radioactivity sedimented as free proteoglycan monomer. Provided that the endogenous and the exogenous link proteins compete on equal terms for binding to the endogenous monomer, most of the endogenous monomers is stabilized by the large excess of exogenous link protein. Thus the result suggests that the low stability of endogenous aggregates at low pH is due to differences in the hyaluronic acid-binding region of the culture proteoglycan rather than an altered link protein.

GENERAL DISCUSSION

Sedimentation in sucrose gradients gives a very rapid separation between monomers and aggregates and can be performed without added carrier. Thus the presence of only free proteoglycan monomer and no aggregates at short times reported in the present paper indicates that the aggregation with hyaluronic acid is an extracellular phenomena. Separate pathways of synthesis and export are then required, since even the intracellular proteoglycan monomers are able to interact with hyaluronic acid added with nasal proteoglycan aggregates to 4 M-guinidinium chloride extracts of the cells.

The present investigation corroborates and extends data by Kimura *et al.* (1979). It is shown directly that formation of hyaluronic acid-proteoglycan aggregates is an extracellular event and that the aggregates formed became rapidly stabilized by link protein. Kimura *et al.* (1979) used competition with proteoglycan monomer to show that formation of link-protein-stabilized aggregates is an extracellular event. The use of suspension cultures, however, provides an advantage, in that

90% of the proteoglycans can be accounted for in the medium already at short chase times compared with about 50% for the proteoglycans from the monolayer culture.

The sedimentation rate of proteoglycan aggregates in a sucrose gradient depends on the number of monomers bound per molecule of hyaluronic acid (Franzén *et al.*, 1981a). Thus the sedimentation patterns show that the size of the aggregates is smaller, and the proportion of bound monomers is lower, at short chase times than at long chase times. At longer chase times, a number of aggregates is large enough to sediment to the bottom at 36 min. There is not, however, a continuous distribution of the aggregates in the sucrose gradient. Rather, the aggregates sedimenting to the bottom of the tube may represent a population distinct from the major population of aggregates. A level of organization above the proteoglycan monomer - hyaluronic acid aggregates, the so called superaggregates, has been postulated (Pita *et al.*, 1975).

The newly formed aggregates were stable to competition with hyaluronic acid oligosaccharides but not stable to competition with hyaluronic acid. This result might be explained if the free monomers were present mainly as monomer-link complexes, as suggested by Kimura *et al.* (1980). An oligosaccharide cannot bind both the monomer and the link protein, whereas the hyaluronic acid can. The monomer-link complex assumingly has a lower affinity for the oligosaccharide than for the hyaluronic acid. In the presence of exogenous oligosaccharide, the monomer without link will bind to the oligosaccharide, whereas the monomer-link complex will bind preferentially to the endogenous hyaluronic acid. In the presence of exogenous hyaluronic acid, however, any free monomer-link complex, as well as the monomer without link, will bind to the exogenous hyaluronic acid.

The proportion of aggregates present at short chase times increased even on incubation with exogenous monomer, although not to the high value of 65% aggregate obtained when medium alone was incubated for 24 h in the absence of cells. This

result supports the presence of monomer-link complexes as discussed above, since the exogenous monomer does not compete with the free endogenous monomer on equal terms. It appears, then, that at least a proportion of the endogenous monomers is present as monomer-link complexes and that some of these complexes bind to hyaluronic acid before the exogenous monomer competes for binding of the link protein. In contrast, no aggregates were formed when exogenous monomer was present during the chase, indicating that the exogenous monomer competed for the newly synthesized link protein at the time of export. In conclusion, the hyaluronic acid molecules, proteoglycan monomers and the link proteins are exported separately and combined in the medium so that monomer-link complexes are formed, which then bind to the hyaluronic acid to form the link-stable aggregates.

In a previous study the proteoglycans isolated by CsCl-density-gradient centrifugation from the medium of chondrocyte cultures were characterized (Björnsson & Heinegård, 1981b). The presence of link proteins in such preparations was demonstrated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Trypsin digestion of link-stable aggregates prepared from cartilage has been used to isolate the hyaluronic acid-binding region of the proteoglycan protein core attached to the hyaluronic acid and protected by the link protein (Heinegård & Hascall, 1974). The complex is resistant even to limit digestion with the enzyme (Heinegård & Axelsson, 1977). If not protected by link proteins, however, the hyaluronic acid-binding fragment is still not appreciably degraded, but its function is lost (Heinegård & Hascall, 1979). In contrast, the proteoglycan isolated from the culture medium was not resistant to trypsin, and was degraded to small peptides by limit digestion with trypsin regardless of whether in link-stabilized aggregate or monomer form (Björnsson & Heinegård, 1981b). The differences between the medium proteoglycan and the nasal cartilage proteoglycan are also demonstrated by the different stabilities of the aggregates at low pH. At pH 4.0 all the endogenous

monomers were displaced from mixed aggregates, whereas most of the exogenous monomer remained aggregated. These results might be interpreted as a relatively low affinity of the hyaluronic acid binding region of the endogenous proteoglycan monomer either to the link protein or to the hyaluronic acid. Thus the lower stability of the proteoglycan aggregates synthesized *in vitro* encountered might result from a difference in the conformation of the hyaluronic acid-binding region. Whether the lower stability of the proteoglycan aggregates from the culture medium represents an earlier state of maturation or an artifact derived from the conditions used *in vitro* is not known.

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Scheme 1

Pulse-chase protocol

6×10^6 cells in 600 μ l/Eppendorf vial



Pulse: 15 min, 20 μ Ci/ml $^{35}\text{SO}_4$

End of pulse: 600 μ l medium containing 0.01 M Na_2SO_4 is added. Centrifuge 30 secs. The medium is removed and the cells suspended in 1200 μ l fresh medium. Repeat once.



Chase: 15 min to 6 h

Centrifuge 30 secs, 50 μ l of the medium is layered on top of a 10-50% sucrose gradient. Centrifuge at 50 000 rpm for 36 min. Freeze the tube and slice in 30 fractions.

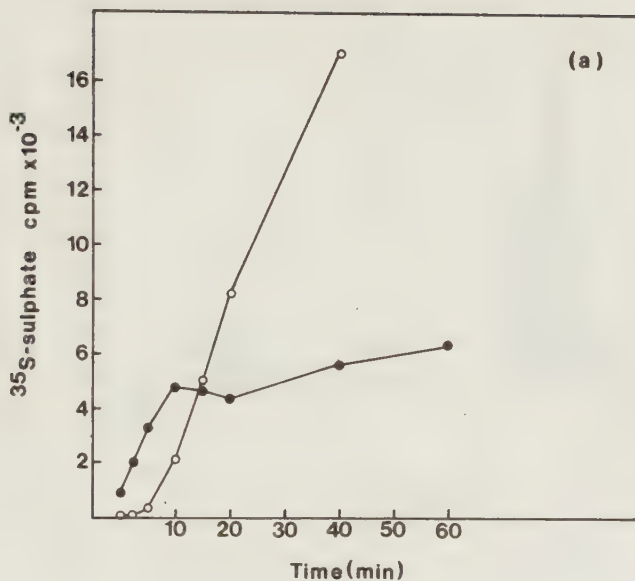


Fig. 1. Incorporation of [35 S]sulphate into proteoglycans isolated by CsCl density gradient centrifugation

Experimental details are given in the text.

O Al fractions prepared from the culture medium

● Al fractions prepared from the 4M-guanidinium chloride extract of the cells.

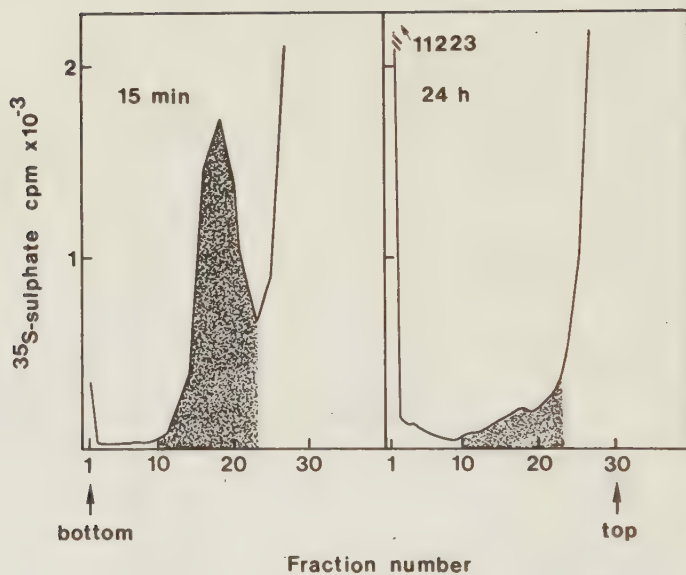


Fig. 2. Proportions of aggregates in the medium from a pulse-chase experiment

Experimental details are given in the text. The chase times are indicated in the Figure. Distribution of $[^{35}\text{S}]$ sulphate in sucrose gradients centrifuged for 3 h at $240\,000\,g_{av}$. The dotted area indicates the position of the monomer.

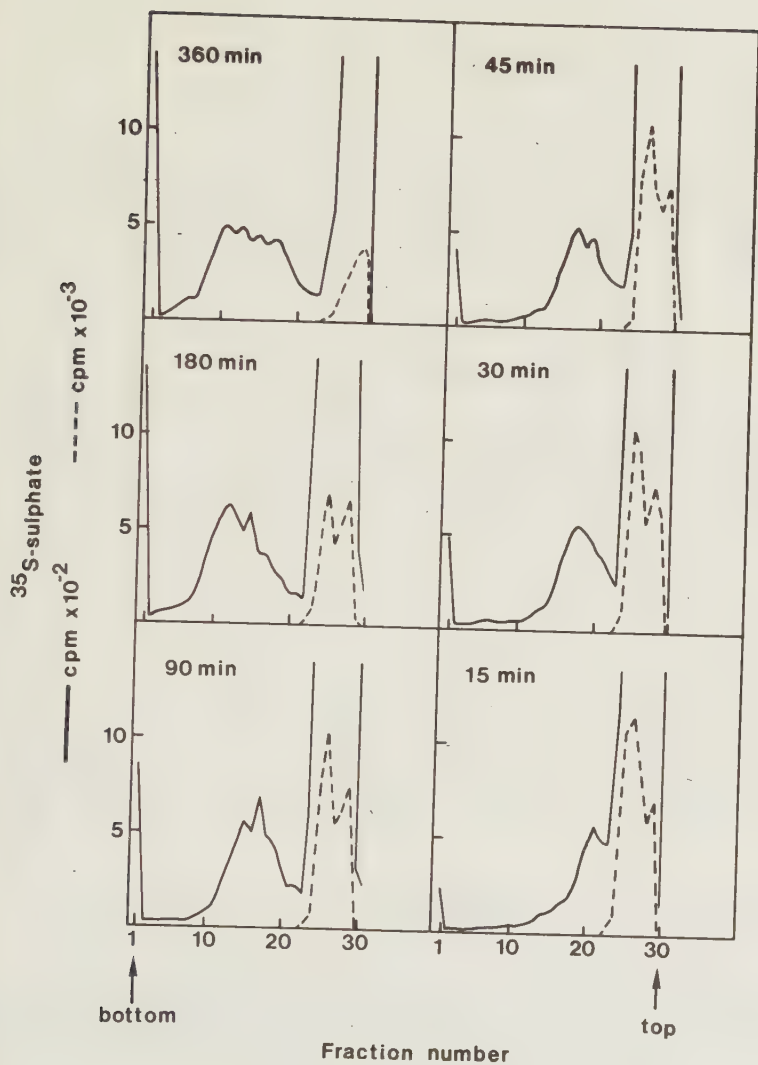


Fig. 3. Size of aggregates in the medium from a pulse-chase experiment. The chase times are indicated in the Figure. Distribution of ^{35}S -sulphate in sucrose gradients centrifuged for 36 min at $240\,000\,g_{av}$. Experimental details are given in the text.

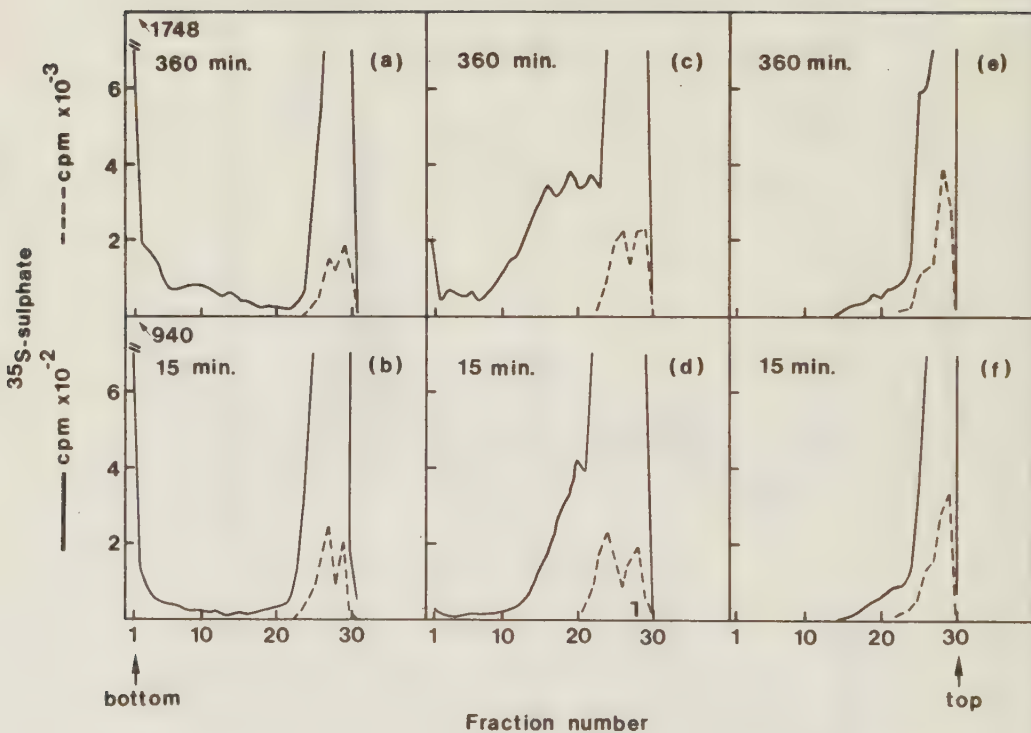


Fig. 4. Competition of aggregates from a pulse-chase experiment with exogenous components. Experimental details are given in the text. Distribution of $[^{35}\text{S}]$ sulphate in sucrose gradients centrifuged for 36 min at $240\,000\text{ g}_{\text{av}}$. The cultures were chased for times indicated in the Figure and the medium incubated with (a,b) exogenous monomer, (c,d) hyaluronic acid oligosaccharides or (e,f) high molecular weight hyaluronic acid.

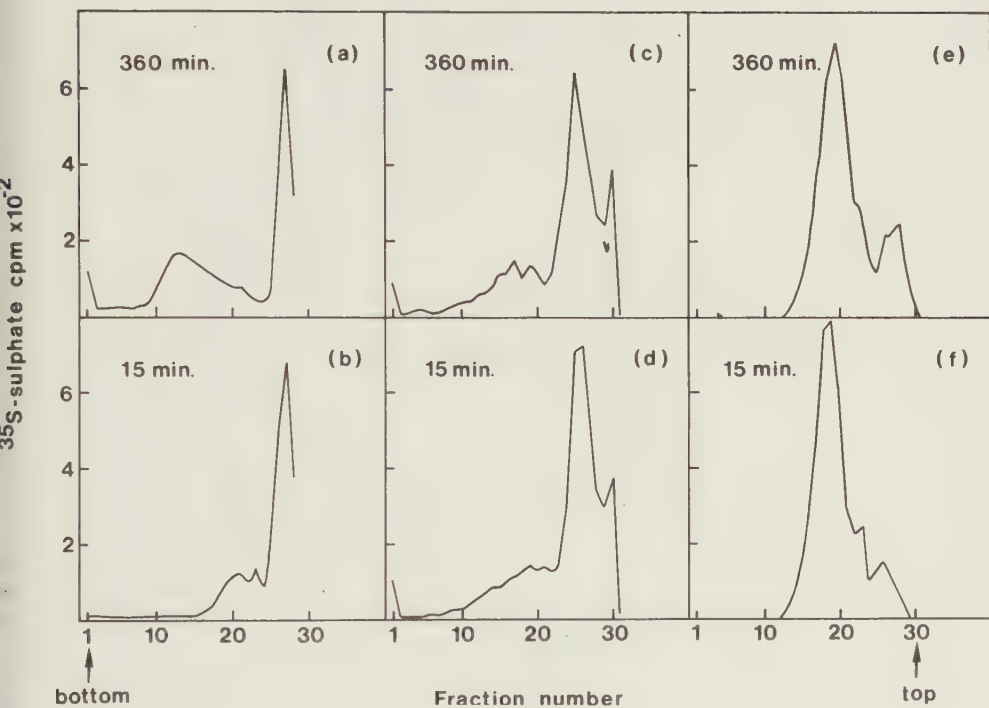


Fig. 5. Dissociation-reassociation of proteoglycans from a pulse-chase experiment. Experimental details are given in the text. The chase times are indicated in the Figure. (a,b) Reassociation without exogenous proteoglycan; distribution of ^{35}S sulphate in sucrose gradients centrifuged for 36 min at $240\,000\,g_{av}$. (c,d) Reassociation with exogenous aggregates; distribution of ^{35}S sulphate in sucrose gradients centrifuged for 1 h at $240\,000\,g_{av}$. (e,f) Reassociation with exogenous monomer; distribution of ^{35}S sulphate in sucrose gradients centrifuged for 3 h at $240\,000\,g_{av}$.

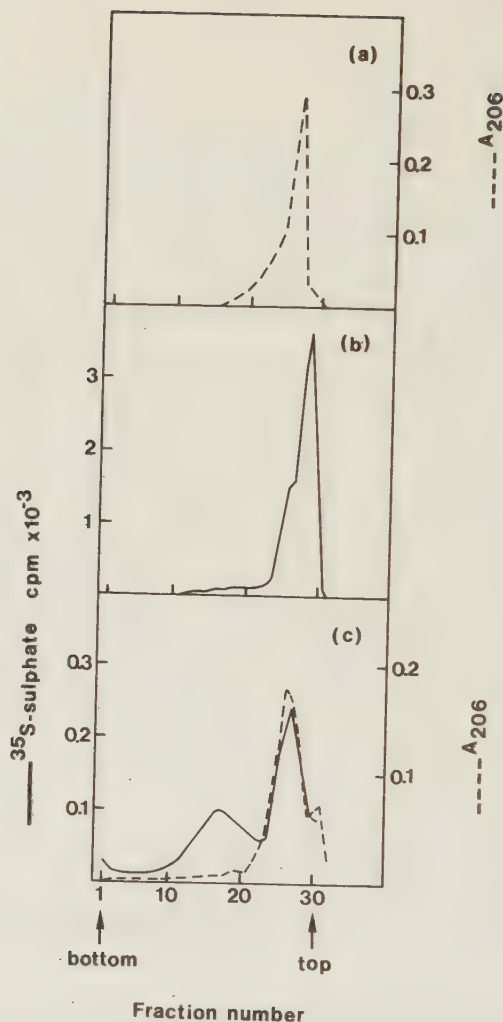


Fig. 6. pH-stability of proteoglycan aggregates prepared from different sources

Experimental details are given in the text. Distribution of [^{35}S]sulphate (—) or absorbance at 206 nm (----) in sucrose gradients centrifuged for 36 min at $240\,000\,g_{av}$. (a) NC-Al-Dl (3 mg/ml) and hyaluronic acid (0.03 mg/ml) incubated for 24 h at pH 4.0. (b) Medium from cultures pulsed with [^{35}S]sulphate and chased for 6 h and the incubated for 24 h at pH 4.0. (c) Medium from cultures pulsed with [^{35}S]sulphate and chased for 6 h and then dissociated-reassociated with exogenous aggregates as in Fig. 5c,d. The reassociated sample was incubated for 24 h at pH 4.0 before analysis.

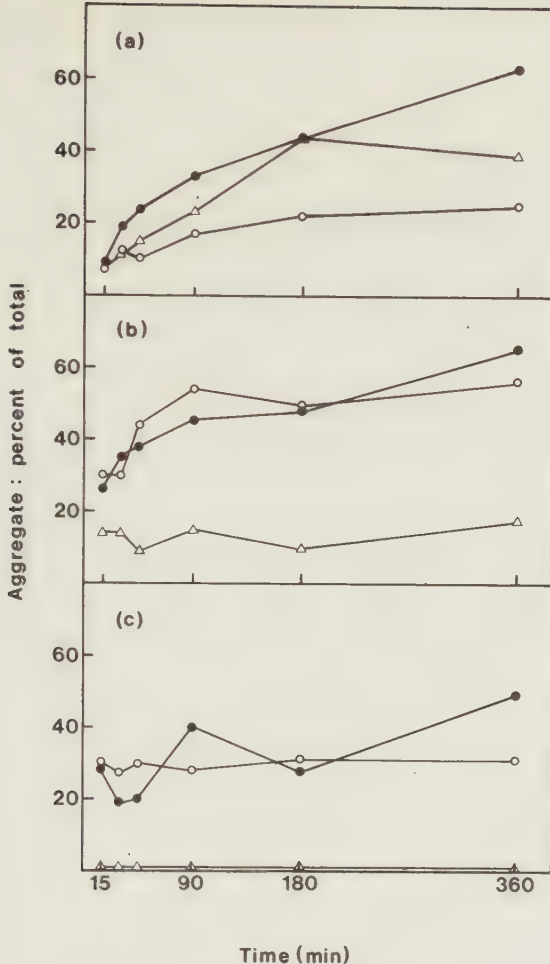


Fig. 7. Proportion of aggregates in medium from a pulse-chase experiment

The chase times are indicated in the Figure.

a) (●) medium analyzed immediately at the end of the chase (○) proportion of aggregates recovered in the bottom fraction of aggregates in fraction 2-23 (Δ) proportion of aggregates recovered in fraction 10-14 of aggregates in fraction 2-23. (The results are obtained from the experiment shown in Fig. 3)

b) (●) medium analyzed after incubation with excess nasal monomer (○) medium analyzed after incubation with excess hyaluronic acid oligosaccharide (Δ) medium analyzed after incubation with excess high molecular weight hyaluronic acid (the results are obtained from the experiments shown in Fig. 4).

c) (●) medium analyzed after dissociation-reassociation without exogenous proteoglycan (○) medium analyzed after dissociation-reassociation with exogenous aggregates (Δ) medium analyzed after dissociation-reassociation with exogenous monomers (the results are obtained from the experiment shown in Fig. 5).

BINDING OF LINK PROTEIN TO PROTEOGLYCAN AND HYALU-
RONIC ACID IN CULTURES OF CHONDROCYTES FROM BOVINE
TRACHEAL CARTILAGE

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SYNOPSIS

Cultures of bovine chondrocytes were pulsed with ^3H -leucine to label the link proteins as well as the proteoglycans secreted to the medium. The distribution of radioactivity in link protein and proteoglycan in aggregate and monomer fractions prepared by zonal rate centrifugation was determined by polyacrylamide gel electrophoresis. The total amount of link protein synthesized in the cultures was determined either as amount of radioactive link protein bound into aggregates after dissociation-reassociation of the medium in the presence of excess exogenous aggregates or as total amount of radioactive link protein present in aggregates and monomers after incubation of the medium with exogenous monomers. In both cases, the molar ratio of endogenous link protein to endogenous proteoglycan monomer was 1:1. The aggregates formed when the medium was incubated for 24 h in the absence of exogenous proteoglycans contained 82 % of the proteoglycans and one link protein per proteoglycan molecule. The not aggregated proteoglycans present in the same sample also contained one link protein per each proteoglycan. Partially assembled aggregates contained more than two molecules of link per proteoglycan molecule. The extra link protein is probably bound only to the hyaluronate, indicating that the newly secreted link protein is free to bind either to the monomers or to the hyaluronic acid. Since all of the proteoglycan monomers at the same time were present as complexes with link protein, it was concluded that only a minor portion of the newly secreted link is bound to hyaluronic acid. The data suggest that the hyaluronic acid, proteoglycan monomer and the link protein are separately exported from the cell. The aggregation is then initiated by binding of the link protein to the proteoglycan monomer. Consequently, aggregates formed by subsequent binding of monomer-link complexes to hyaluronic acid are link-stable.

INTRODUCTION

The majority of proteoglycans present in cartilage are aggregated to hyaluronic acid (for references, see Hascall & Heinegård, 1979). This aggregation occurs extracellularly as shown in chondrocyte cell cultures (Kimura *et al.*, 1979; Kimura *et al.*, 1980; Björnsson & Heinegård, 1981c). The formation of large, link-stable, aggregates was inhibited by addition of hyaluronic acid oligosaccharides (25-50 monosaccharides) to the medium of monolayer cultures of rat chondrosarcoma chondrocytes (Kimura *et al.*, 1979). Such oligosaccharides formed ternary complexes with the newly secreted proteoglycan monomers and link proteins, displacing all not link-stabilized monomers from the endogenous hyaluronic acid. It was suggested that link-stable aggregates are formed outside the cells, since all newly secreted proteoglycans were bound into such ternary complexes with the exogenous hyaluronic acid oligosaccharides. Corroborating evidence was obtained (Björnsson & Heinegård, 1981c) using zonal rate centrifugation in sucrose gradients to separate newly secreted ³⁵S-sulphate labelled proteoglycan monomers and aggregates synthesized in suspension cultures of bovine chondrocytes. Insignificant amount of aggregate were found at short chase times (15 min) although both link-stable and link-free aggregates are stable under the conditions of sedimentation (Franzén *et al.*, 1981b). It was concluded that proteoglycan monomers and hyaluronic acid are exported separately. The size of the aggregates increased as the proportion of proteoglycans bound into aggregates increased, indicating that the extracellular assembly occurs at a random. In a recent paper Kimura *et al.* (1980) presented evidence that the molar ratio of link protein to proteoglycan is 1:1 in the link-stable aggregates. Moreover, the link protein appeared to bind to the monomers already before aggregation. To test this possibility, competition experiments were performed with exogenous hyaluronic acid or hyaluronic acid oligosaccharides (Björnsson & Heinegård, 1981c). The newly assembled aggregates were stable when incubated with oligosaccharides but not when incubated with high molecular weight hyaluronic acid. In addition, the

proportion of endogenous proteoglycan monomers bound into link-stable aggregates increased on incubation with exogenous monomer. The behaviour of the endogenous monomer in these competition experiments could only be explained if it was present as complex with link protein already prior to aggregation.

In contrast to proteoglycans derived from the rat chondrosarcoma the bovine proteoglycans contain the keratan sulphate enriched region (Heinegård & Axelsson, 1977; Björnsson & Heinegård, 1981b) as well as both link proteins (Keiser *et al.*, 1972; Björnsson & Heinegård, 1981b).

The present study was undertaken to determine the sequence of events during binding of link protein in proteoglycan aggregates formed in cultures of bovine chondrocytes labelled with ³H-leucine.

EXPERIMENTAL

Materials. Collagenase CLS II obtained from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). Ham's F12 medium was from GIBCO (Grand Island, NY, U.S.A.). Benzoylpenicillin was from Astra (Stockholm, Sweden). Streptomycin sulphate was from Merck (Darmstadt, Germany). Polypropylene vials (2.2 ml, no. 3812) were obtained from Eppendorf (Hamburg, Germany). ³H-leucine (65 Ci/mmol) was from The Radiochemical Centre (Amersham, Bucks, U.K.). Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Hyaluronic acid (Healon) was a gift from Pharmacia Fine Chemicals (Uppsala, Sweden). Dextran sulphate and Sepharose 2B were purchased from Pharmacia. Instagel was from Packard Instrument Co. (La Grange, IL, U.S.A.).

Proteoglycan aggregates (NC-A1 fraction) monomers (NC-A1-D1 fraction) and link proteins (NC-A1-D4 fraction) were prepared from bovine nasal cartilage as described previously (Heinegård, 1972; Heinegård & Hascall, 1979).

Cell culture methods

Preparation of cell cultures. Chondrocytes were isolated from foetal bovine tracheas as described previously (Björnsson & Heinegård, 1981a). After incubation for 1-2 days at 37°C, the cells were collected by centrifugation and suspended in Ham's F12 medium (without streptomycin sulphate). The cell suspension was pipetted into Eppendorf vials and incubated at 10×10^6 cells/ml on an Adams nutator with orbital motion for 1 h before being labelled with ^3H -leucine.

Proportion of link protein to proteoglycan. The cells (20×10^6 in 2 ml) were pulsed with ^3H -leucine (20 $\mu\text{Ci/ml}$) for 3 h. The pulse was terminated by pelleting the cells for 30 s in an Eppendorf centrifuge (model 5412) operated with low voltage (90 V). One portion of the medium (100 μl) was mixed with one volume 8 M-guanidinium chloride and NC-Al (300 μg) was added to the sample. The dissociated proteoglycans were dialysed to associative conditions (0.15 M NaCl, 5 mM- NaH_2PO_4 , pH 7.0) in a microdialysis chamber (Franzén *et al.*, 1981b). The re-associated proteoglycans were fractionated by rate zonal centrifugation in sucrose gradients (Björnsson & Heinegård, 1981c) performed as described below. The fractions containing aggregates, monomers and non-proteoglycan proteins were separately pooled as indicated in Fig. 1, extensively dialysed against 0.1 % (w/v) sodium dodecyl sulphate at 4°C and subsequently lyophilized. The samples were electrophoresed on polyacrylamide gels to determine the distribution of radioactivity in link protein and proteoglycan.

Binding of link protein to proteoglycan monomer. Other portions (200 μl) of the medium incubated for 24 h at 4°C with or without added NC-Al-D1 (200 μg). The samples were fractionated into aggregates, monomers and non-proteoglycan proteins by zonal rate centrifugation in sucrose gradients. Portions of the pooled (not dialysed) fractions from the gradients were subjected to polyacrylamide gel electrophoresis to determine the distribution of radioactivity in link protein and proteoglycan.

Binding of protein to hyaluronic acid. Cultures (20×10^6 cells in 2 ml) were pulsed for 3 h with ^3H -leucine ($20 \mu\text{Ci/ml}$) to ensure uniform labelling of the proteoglycans and link proteins. The cells were pelleted, the medium was discarded and the pulse was continued for 1 h with fresh medium containing isotope. Portions ($6 \times 200 \mu\text{l}$) of the medium were fractionated by zonal rate centrifugation in sucrose gradients immediately at the end of the 1 h pulse. The bottom fraction and fractions 16-20, respectively, from the six gradients were pooled and extensively dialysed against 0.1 % (w/v) sodium dodecyl sulphate. Other portions ($6 \times 200 \mu\text{l}$) of the medium were incubated for 16 h at 37°C before zonal rate centrifugation. Fractions were pooled and dialysed as above. The proportions of link proteins in the aggregate and monomer fractions were determined by polyacrylamide gel electrophoresis.

Analytical methods

Zonal rate centrifugation in sucrose gradients. Linear gradients (3.6 ml) with 10-50 % (w/v) sucrose in $0.25 \text{ M-Cs}_2\text{SO}_4/5 \text{ mM-sodium phosphate buffer}$, pH 7.0, were prepared in 4.2 ml MSE polycarbonate tubes as described elsewhere (Franzén *et al.*, 1981a; Björnsson & Heinegård, 1981c). Samples ($200 \mu\text{l}$) were carefully layered on top of the gradients. After centrifugation for 3 h in an MSE $6 \times 4.2 \text{ ml}$ swing-out rotor at $240\,000 g_{\text{av}}$, (r_{av} 88 mm) and 20°C , the aggregates have sedimented to the bottom, the monomers to the middle and the non-proteoglycan proteins remain at the top of the gradient. The separation between proteoglycan monomers and non-proteoglycan proteins is improved in the presence of cesium (Franzén *et al.*, 1981a). The gradients were frozen and sliced as previously described (Björnsson & Heinegård, 1981c).

Polyacrylamide gel electrophoresis. Electrophoresis was performed in the presence of sodium dodecyl sulphate as described by Neville (1971). Gels were cast in Perspex tubes ($100 \times 8 \text{ mm}$, inner diameter) with 4.1 % acrylamide and 0.7 % N,N-diallyltartardiamide (spacer gel, 10 mm) or 9.7 % acrylamide and 0.4 %

N,N-diallyltartardiamide (separation gel, 80 mm). Samples for electrophoresis with added link protein carrier (20 μ g) were reduced by boiling in 5 % (w/v) 2-mercaptoethanol and 2 % (w/v) sodium dodecyl sulphate. The samples (0.5 ml) were applied to the gel in the upper reservoir buffer, pH 8.64, containing 5 % (w/v) sucrose and trace amounts of bromphenol blue as marker. The Cs_2SO_4 present in the non dialysed samples did not affect the stacking of the proteins since the upper gel buffer is buffered with H_2SO_4 (Neville, 1971). Electrophoresis was done at 5 mA/tube at constant current for 5 h. The gels were stained with Coomassie Brilliant Blue R250 and the positions of the link proteins were marked with surgical wire. The gels were sliced frozen with a Mickle (Gomshall, Surrey, England) gel slicer. The spacer gel (not sliced) and the slices (1 mm) were dissolved in 1 ml of 2 % periodic acid and counted in 10 ml of Instagel using a Packard 2650 scintillation counter.

RESULTS AND DISCUSSION

Proportion of link protein to proteoglycan monomer. Proteoglycans from the medium of ^3H -leucine-labelled cultures dissociated-reassociated in the presence of bovine nasal cartilage proteoglycan aggregates. After reassociation, the proteoglycans were fractionated by zonal rate centrifugation, Fig. 1. The fractions containing aggregates, monomers and the non-proteoglycan proteins, respectively, were pooled, as indicated in Fig. 1, and subjected to sodium dodecyl sulphate gel electrophoresis, Fig. 2a, b, c. The proteoglycans are caught in the spacer gel and do not penetrate the more concentrated separation gel. The proteoglycans can be quantitatively recovered from the spacer gel after staining and destaining (data not shown). The link proteins migrate with an R_f of 0.51. The aggregate fraction contained a radioactive peak at the position of the carrier link protein, Fig. 2a. The radioactivity in the link protein (shaded area) constituted 28 % of the combined radioactivity in this peak and in the spacer gel, Table 1. The monomer fraction gave little radioactivity in

the link region, Fig. 2b, *i. e.* 2.8 % of the combined radioactivity, Table 1. The top fraction, containing the proteins not bound to aggregates or monomers, gave a major radioactive peak with a mobility similar to that of the small link protein, Fig. 2c. Since this protein was not recovered with the reassociated aggregates, it is probably not related to the link proteins. It appears that the proteoglycan aggregate contains one link protein per bound proteoglycan (Heinegård & Hascall, 1974; Kimura *et al.*, 1980). If so, 19 % of the leucine content of an aggregate would represent link protein, assuming an average molecular weight of 2.5×10^6 (Hascall & Sajdera, 1970), a protein content of 7.3 % and a leucine content of 70 residues per 1000 amino acids for the proteoglycan monomer (Björnsson & Heinegård, 1981b) and a mean molecular weight of 43 250 (46 000 and 40 500, respectively), a protein content of 94 % and a leucine content of 79 residues per 1000 amino acids for the link proteins (Baker & Caterson, 1979).

The aggregate fraction then contained about $1.5 \text{ }^3\text{H}$ -leucine labelled link proteins per ^3H -leucine labelled proteoglycan monomer. Almost all radioactive link protein (92 %) was recovered in this fraction. In contrast, the aggregate fraction contained only 46 % of the radioactive monomers, Table 1. The reassociation of the newly synthesized proteoglycans into aggregates is only partial as reported earlier (Björnsson & Heinegård, 1981c). Most of the link protein was bound in stable aggregates and only a minor portion sedimented with the monomers. In fact the low amount of link protein in the monomer fraction indicate that all link protein synthesized in the cultures were accounted for in this fraction and the aggregate fraction. The total proportion of radioactive link protein to radioactive proteoglycans was 16.5 %, Table 1. Taking into account the lower recovery (76 %) of the aggregate fraction upon electrophoresis and assuming non-specific losses, 18 % of the radioactivity was in link protein, which is close to one molecule of link per proteoglycan monomer.

Binding of link protein to proteoglycan monomer. Medium from cultures that had been labelled with ^3H -leucine was incubated for 24 h at 4°C . The labelled proteins were fractionated by zonal rate centrifugation into aggregates, monomers and non-proteoglycan proteins, Fig. 3a. Contents of ^3H -leucine labelled link proteins and proteoglycans were determined by SDS-gel electrophoresis as discussed above. After 24 h of incubation 82 % of the proteoglycans were recovered in the aggregate fraction, Table 2. Almost all ^3H -labelled proteins, penetrating into the separation gel, were recovered at the position of the link protein carrier, Fig. 4a, and constituted 17.5 % of the amount of ^3H -leucine incorporated into proteoglycans and link together, Table 2. This is close to value of one link protein per proteoglycan expected in stable aggregates. A small amount of another protein of higher molecular weight (R_f 0.27) was also present. In the monomer fraction, 20 % of the radioactivity was recovered in the link protein band, Fig. 4b, Table 2, indicating that the monomer fraction contains link protein bound to the proteoglycan in a monomer-link complex. The recovery of the radioactivity from the gel representing the monomer fraction, however, was only 36 %, Table 2. It is likely that the large amounts of unincorporated ^3H -leucine present at the top of the sucrose gradient, contaminated the monomer fraction. Such unincorporated isotope is lost during electrophoresis, giving the apparent low recovery of ^3H -leucine in this fraction. The radioactivity in the aggregate fraction, however, was quantitatively recovered on electrophoresis, indicating that little macromolecular radioactivity is lost during the procedure. Both link proteins found in bovine tracheal cartilage (Paulsson & Heinegård, 1979) were present in the aggregate as well as the monomer fractions. The total proportion of radioactivity recovered as link protein in the aggregate and monomer fractions was 18.1 % or close to one link protein per proteoglycan monomer.

The top fraction of the sucrose gradient contains proteins not bound to the proteoglycans, Fig. 4c. The dominating protein has a mobility (R_f 0.57) slightly higher than the small link pro-

tein (R_f 0.54). For comparison the unfractionated medium was electrophoresed, Fig. 4d. A radioactive peak corresponding to the large link protein (R_f 0.51) was present. This peak was not present in the top fraction of the sucrose gradient, indicating that most or all of the link proteins were bound to proteoglycan aggregates or monomers. The presence of the small link protein could not be demonstrated, probably because the small link protein is poorly resolved from the much larger preceeding peak.

Another portion of the same medium was incubated with excess monomer for 24 h at 4°C and analysed as described above. The presence of exogenous monomer inhibited further aggregation, and the majority of the radioactive proteoglycans (74 %) was recovered as monomers in the sucrose gradient, Fig. 3b and Table 2. The aggregate fraction gave only one radioactive peak on polyacrylamide gel electrophoresis, having the same mobility as the link proteins, Fig. 5a. This peak contained 42 % of the combined radioactivity of link protein and proteoglycan, Table 2. In contrast, only 9.2 % of the radioactivity in the monomer fraction was link protein, Fig. 5b and Table 2. The total proportion of radioactivity recovered as link was 20.1 %, Table 2. Recently, binding of exogenous monomers to free binding sites in endogenous aggregates was demonstrated (Björnsson & Heinegård, 1981c). The present results, in addition, suggest that the exogenous monomer is stabilized by endogenous link protein, resulting in the high proportion of radioactive link protein (42 %) in aggregates. The excess of link protein in aggregates, approximately two link proteins per endogenous proteoglycan monomer, is probably derived from endogenous monomer-link complexes. The ratio of radioactive link in the monomer fraction is low (9.2 %), indicating that the exogenous monomer has competed with the endogenous monomer-link complexes for binding of the link protein. The equilibrium between exogenous and endogenous monomers for binding of link protein is apparently slow, since the proportion of link stable endogenous monomers incorporated in aggregates increased even during incubation in the presence of excess exogenous monomer

competing for binding of the link protein (Björnsson & Heinegård, 1981c).

Following incubation with excess monomer, the top fraction of the sucrose gradient still contains a major radioactive peak with an R_f slightly higher than that of the link proteins, Fig. 5c. This protein does apparently not have any affinity for proteoglycan monomers, since the amount of radioactivity in the peak is similar with or without addition of exogenous monomer (3857 and 4138 dpm, respectively). Any excess functional link protein present would be recovered in the aggregate or the monomer fraction (as monomer-link complex) on addition of exogenous monomer. The overall ratio of radioactive link protein to radioactive proteoglycan was similar with and without exogenous monomer, however, in both cases approximately 1:1.

Binding of link protein to hyaluronic acid. Chondrocytes were pulse-labelled for three hours with ^3H -leucine to equilibrate the intracellular pool, the medium was changed and the pulse was continued for 1 h. The proteoglycans secreted during the 1 h pulse were fractionated into aggregates and monomers by sedimentation in sucrose gradients (not shown), either directly or following incubation for 16 h at 37°C . The aggregate fraction and a portion of the monomer fraction (fraction 16-20) was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis to determine the proportion of radioactivity present in link protein, Table 3. After a one hour pulse only a low proportion of the newly secreted labelled proteoglycans are assembled into aggregates, Table 3 (Björnsson & Heinegård, 1981c). The amount of ^3H -labelled aggregates increased more than tenfold when the medium was incubated in the absence of cells for 16 h, Table 3. The proportion of radioactive link protein in aggregates assembled during the one hour pulse was 54 %, corresponding to 2.8 molecules of link per proteoglycan monomer. In the same sample all the free monomers were present as monomer-link complexes as suggested by the proportion of radioactive link protein present in this fraction (19 %). In contrast, after incubation for 16 h to obtain maximal aggregation, the aggregates contained the expected ratio of one

(1.2) link per proteoglycan monomer, Table 3. In the monomer fraction there was slightly less than one link protein (0.79) per proteoglycan.

It is likely that the high proportion of link protein in the aggregates recovered after 1 h of pulse is due to link protein bound to the hyaluronic acid but not to proteoglycan monomer. In support, it has been shown that link proteins are capable of interaction with hyaluronic acid alone (Heinegård & Hascall, 1974; Caterson & Baker, 1978). The extra link protein bound to the aggregates indicates that link protein is secreted separately from proteoglycans and can bind either to hyaluronic acid or proteoglycan monomer. Corroborating evidence (Björnsson & Heinegård, 1981c) is that the presence of a large excess exogenous monomer during the chase was found to inhibit all aggregation. In contrast, when exogenous monomer was added at later times, the aggregation continued although less labelled aggregates were found. The results are readily explained, if the exogenous monomer added at the beginning of the chase was able to compete for binding of the newly secreted free link protein. When exogenous monomer was added after the chase, however, the endogenous monomer was in complex with link protein. Consequently, exogenous monomer did not compete for binding to hyaluronic acid on equal terms with endogenous monomer indicating the presence of proteoglycan-link precursors.

GENERAL DISCUSSION

Two different approaches were tried to determine the ratio of link protein to proteoglycans synthesized in chondrocyte cultures. When dissociation-reassociation in the presence of exogenous aggregates was used to bind all functional link protein in aggregates, a ratio of one (0.95) link protein per proteoglycan molecule was found. A large proportion of the endogenous proteoglycan was, however, recovered as monomer which might suggest an incomplete renaturation of the endogenous proteoglycans upon dialysis to associative conditions. An additio-

nal component, with a mobility close to that of the link proteins was found in the non-proteoglycan protein fraction, possibly representing non-functional link protein. In another experiment addition of proteoglycan monomers was used to bind any excess link protein present in the medium. The ratio of endogenous link protein to proteoglycan was close to 1:1. The non-proteoglycan protein fraction again contained the prominent peak at the position of the link protein carrier indicating that this protein, even in the native stage, was not able to bind to the proteoglycans. The link protein is thus not synthesized in molar excess relative to proteoglycan monomer. In this type of cultures, in contrast to cultures of rat chondrosarcoma chondrocytes (Kimura *et al.*, 1980). None of the excess link protein synthesized in chondrosarcoma cultures was recovered in aggregates prepared after CsCl gradient centrifugation of the medium. Probably, the binding of link protein to hyaluronic acid, in the absence of monomers, may not be stable to the high salt conditions during preparation.

Kimura *et al.* (1980) suggested that link-stable aggregates are formed via a monomer-link complex intermediate, which is in agreement with the results presented here. They found that the endogenous link protein could not be displaced from the monomer-link complex by addition of exogenous monomer. At the much higher concentration of exogenous monomer used in the present work, at least a portion of the endogenous link protein in aggregates with exogenous monomer, was displaced from the endogenous monomer-link complexes.

Only a minor portion of the newly secreted proteoglycan monomers was recovered in aggregates, link-free or link-stable, although the link protein present in the medium was already bound to the monomers or partially assembled aggregates. The excess link protein present in such aggregates indicate that the link protein is secreted separately from the proteoglycans. It is likely, then, that the first event in formation of link-stable aggregates is the binding of link, either to monomers or to hyaluronic acid. The proportion of link protein bound to

the hyaluronic acid will reflect the respective binding constant of the link protein-hyaluronic acid complex versus the link protein-monomer complex, as well as the ratio of available hyaluronic acid to monomer. The actual proportion of link bound alone to the hyaluronic acid is probably low, since the majority of not aggregated proteoglycan monomer in the same sample was present as complex with link protein. The link protein bound alone to hyaluronic acid will probably equilibrate with the proteoglycan monomer pool to form monomer-link complexes.

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Table 1. Distribution of radioactivity in link protein and proteoglycan monomer in culture medium dissociated-reassociated with exogenous aggregates. Experimental details are given in the text. Proteoglycan aggregate and monomer fractions were prepared by centrifugation in sucrose gradients. Link proteins and proteoglycans were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Molar ratios of link to monomer were calculated assuming that 19% of the leucin content is in link if one link protein is bound per proteoglycan monomer.

Fraction	Recovery after electrophoresis (%)	Proteoglycan		Link (dpm)	Link		Link/Monomer (molar ratio)
		(dpm)	(%)		Link+Proteoglycan (%)		
Aggregate	76	4974	46	1961	28.3		1.5
Monomer	102	5813	54	165	2.8		0.15
Total		10787	100	2126	16.5		0.87

Table 2. Distribution of radioactivity in link protein and proteoglycan monomer in culture medium incubated for 24 h at 4°C without (a) or with (b) added proteoglycan monomer (1mg/ml).

Experimental details are given in the text. Proteoglycan aggregate and monomer fractions were prepared by centrifugation in sucrose gradients. Link proteins and proteoglycans were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Molar ratios were calculated as described in Table 1.

Fraction	Recovery after electrophoresis (%)	Proteoglycan (dpm)	(%)	Link (dpm)	<u>Link Link+Proteoglycan</u> (%)	Link/Monomer (molar ratio)
<u>a</u>						
Aggregate	109	12945	82	2754	17.5	0.92
Monomer	36	2887	18	746	20.0	1.05
Total		15832	100	3500	18.1	0.95
<u>b</u>						
Aggregate	107	6149	26	4553	42.0	2.20
Monomer	62	17883	74	1741	9.2	0.48
Total		24032	100	6294	20.1	1.10

Table 3. Distribution of radioactivity in link protein and proteoglycan monomer in medium from cultures labelled for 1 h with ^3H -leucin and analyzed directly (a) or after incubation for 16 h at 37°C (b).

Experimental details are given in the text. Proteoglycan aggregate and monomer fractions were prepared by centrifugation in sucrose gradients. Link proteins and proteoglycans were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Molar ratios were calculated as described in Table 1.

Fraction	Recovery after electrophoresis (%)	Proteoglycan (dpm)	Link (dpm)	Link Link+Proteoglycan (%)	Link/Monomer (molar ratio)
<u>a</u>					
Aggregate	93	1816	2123	54	2.8
Monomer (fraction 16-20)	108	22266	5139	19	1.0
<u>b</u>					
Aggregate	111	35719	10499	23	1.2
Monomer (fraction 16-20)	106	11955	2050	15	0.8

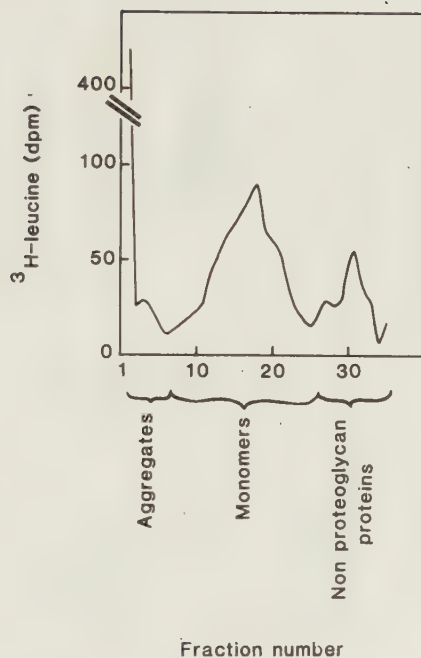


Fig.1. Distribution of ³H-leucine after zonal rate centrifugation of medium dissociated-reassociated with exogenous proteoglycans (NA-A1 fraction, 3 mg/ml). Experimental details are given in the text. The fractions were pooled as indicated.

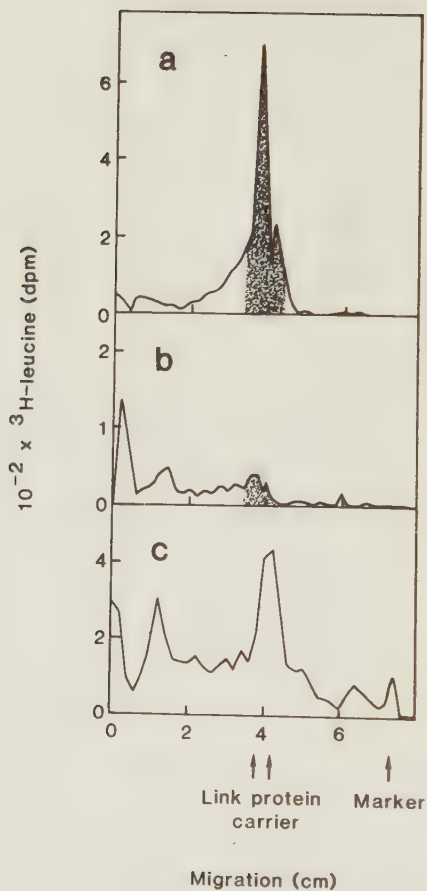


Fig.2. Sodium dodecyl sulphate polyacryl-
amide gel electrophoresis of the fractions
pooled after zonal rate centrifugation, fig.1.
 Experimental details are given in the text.
 The arrows indicate the position of the link
 protein carrier. Corresponding fractions
 (shaded) were used for calculation of radio-
 activity in link protein. (a) aggregate
 fraction (b) monomer fraction (c) non pro-
 teoglycan protein fraction

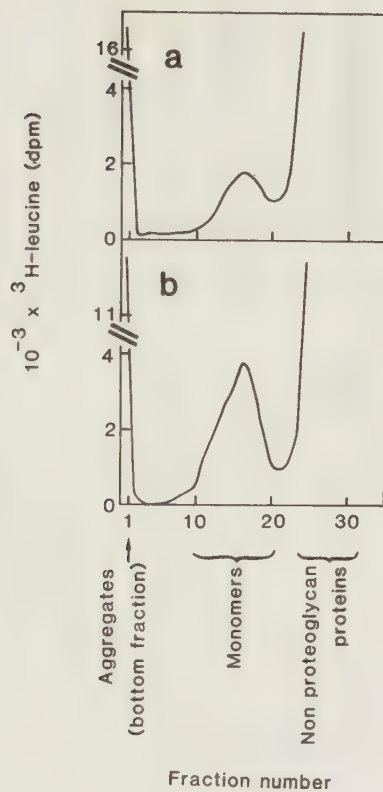


Fig.3. Distribution of ${}^3\text{H-leucine}$ after zonal rate centrifugation of medium incubated for 24 h at 4°C without (a) or with (b) exogenous proteoglycan monomers (NC-A1-D1 fraction, 1 mg/ml). Experimental details are given in the text. The fractions were pooled as indicated.

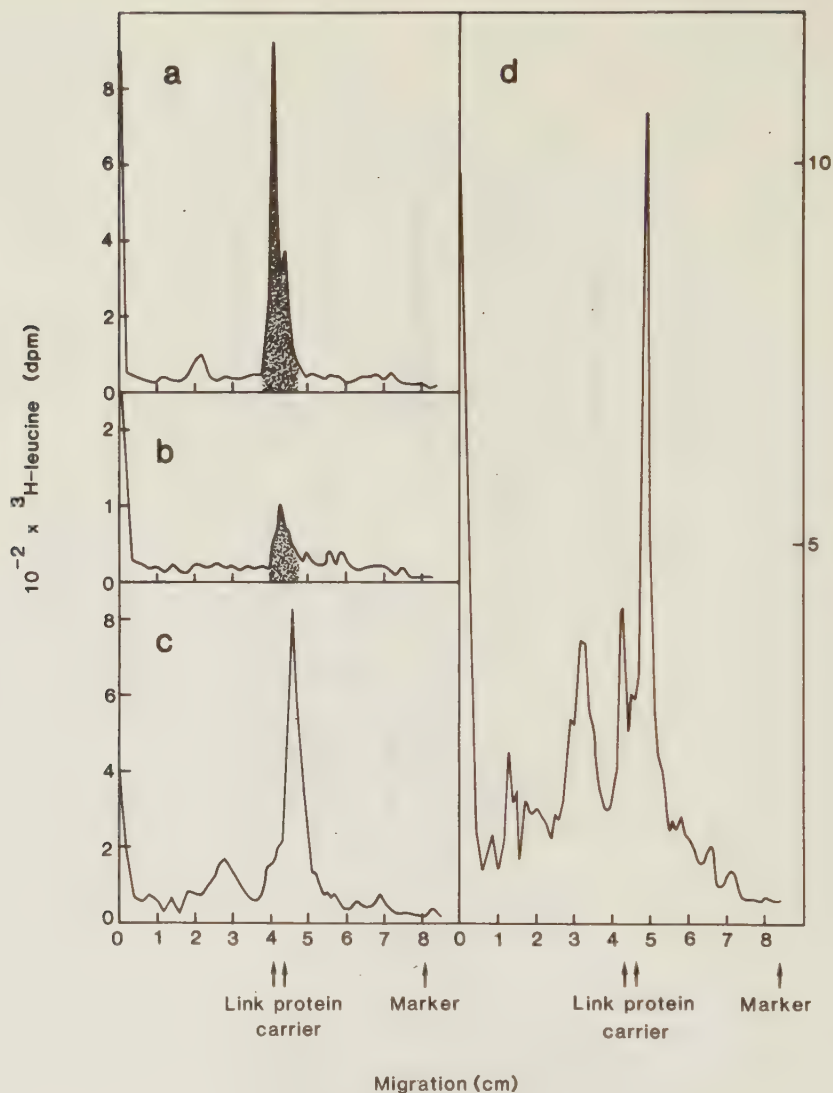


Fig.4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of the fractions pooled after zonal rate centrifugation, fig. 3a and of the unfractionated medium. Experimental details are given in the text. The arrows indicate the position of the link protein carrier. Corresponding fractions (shaded) were used for calculation of radioactivity in link protein. (a) aggregate fraction (b) monomer fraction (c) non proteoglycan protein fraction (d) unfractionated medium

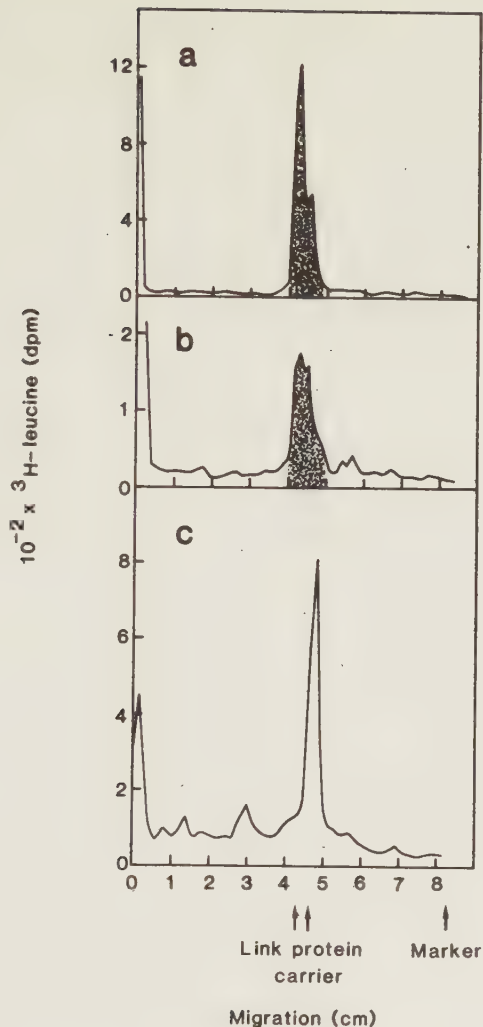


Fig.5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of the fractions pooled after zonal rate centrifugation, fig 3b. Experimental details are given in the text. The arrows indicate the position of the link protein carrier. Corresponding fractions (shaded) were used for calculation of radio-activity in link protein. (a) aggregate fraction (b) monomer fraction (c) non proteoglycan protein fraction

